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EXAMINER	
ASHEN, JON BENJAMIN	
ART UNIT	**PAPER NUMBER**
1635	

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Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No. 10/688,706	Applicant(s) BROSCHAT ET AL.	
	Examiner Jon B. Ashen	Art Unit 1635	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☐ Responsive to communication(s) filed on _____.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) 1-32 is/are pending in the application.

4a) Of the above claim(s) 1-18,21-27 and 29-32 is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) 19,20 and 28 is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☒ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: _____.

DETAILED ACTION

Election/Restrictions

1. Restriction to one of the following inventions is required under 35 U.S.C. 121:

   I. Claims 1-18, drawn to an antisense compound targeted to a nucleic acid molecule encoding GFAT, classified in class 536, subclass 24.5.

   II. Claims 19-32, drawn to a method of treatment comprising inhibiting the expression of mPGES-1 in cells, tissues or a human, classified in class 514, subclass 44.

2. Claims 4-7 are subject to an additional restriction since the sequences listed as SEQ ID Nos: 1-3063 are not considered to be a proper genus/Markush. See MPEP 803.02 - PRACTICE RE MARKUSH-TYPE CLAIMS - If the members of the Markush group are sufficiently few in number or so closely related that a search and examination of the entire claim can be made without serious burden, the examiner must examine all the members of the Markush group in the claim on the merits, even though they are directed to independent and distinct inventions. In such a case, the examiner will not follow the procedure described below and will not require restriction. Since the decisions in In re Weber, 580 F.2d 455, 198 USPQ 328 (CCPA 1978) and In re Haas, 580 F.2d 461, 198 USPQ 334 (CCPA 1978), it is improper for the Office to refuse to examine that which applicants regard as their invention, unless the subject matter in a claim lacks unity of invention. In re Harnish, 631 F.2d 716, 206 USPQ 300 (CCPA 1980); and Ex parte Hozumi, 3 USPQ2d 1059 (Bd. Pat. App. & Int. 1984). Broadly, unity of invention

exists where compounds included within a Markush group (1) share a common utility, and (2) share a substantial structural feature disclosed as being essential to that utility.

Claims 4-7 specifically claim antisense sequences as listed, which are targeted to and modulate the expression of GFAT. Although the antisense sequences claimed each target and modulate expression of GFAT, the instant antisense sequences are considered to be unrelated, since each antisense sequence claimed is structurally and functionally independent and distinct for the following reasons: each antisense sequence has a unique nucleotide sequence, each antisense sequence targets a different and specific region of a GFAT nucleic acid, and absent evidence to the contrary, each antisense, upon binding to a GFAT nucleic acid, is expected to functionally modulate (increase or decrease) the expression of GFAT to varying degrees. As such the Markush/genus of antisense sequences in claims 4-7 are not considered to constitute a proper genus, and are therefore subject to restriction.

Furthermore, a search of more than one (1) of the antisense sequences claimed in claims 4-7 presents an undue burden on the Patent and Trademark Office due to the complex nature of the search and corresponding examination of more than one (1) of the claimed antisense sequences. MPEP 808.02 states in part: Where the related inventions as claimed are shown to be distinct under the criteria of MPEP 806.05(C) - 806.05(i), the examiner, in order to establish reasons for insisting upon restriction, must shown by appropriate explanation one of the following:

(C) A different field of search: Where it is necessary to search for one of the distinct subjects in places where no pertinent art to the other subject exists, a different field of search is shown, even though the two are classified together.

It is noted that a search of the available sequence databases produces a listing of references disclosing the sequence most similar to the query sequence. This is the "place" where the examiner searches for prior art. The prior art relating to another query sequence will not be found in this "place"- a different listing of references must be generated and searched by the examiner. Thus a different search is shown, and restriction is proper.

In view of the foregoing, one (1) antisense sequence is considered to be a reasonable number of sequences for examination. Accordingly, applicant is required to elect one (1) sequence from claims 4-7 that will be examined on the merits. Note that this is not a species election.

The inventions are distinct, each from the other because of the following reasons:

3. Inventions I and II are related as product and process of use. The inventions can be shown to be distinct if either or both of the following can be shown: (1) the process for using the product as claimed can be practiced with another materially different product or (2) the product as claimed can be used in a materially different process of using that product (MPEP § 806.05(h)). Invention I is drawn to an antisense compound targeted to a nucleic acid molecule encoding GFAT. Invention II is drawn to a method of treatment comprising inhibiting the expression of mPGES1 in cells, tissues or a

human. In the instant case the product as claimed can be used in a materially different process of using that product such as a hybridization probe to elucidate cell or tissue specific gene expression of GFAT.

Furthermore, searching inventions I and II together would impose a serious search burden. In the instant case, prior art searches of an compound sequence and a method of using said compound sequence are not coextensive. Search of each of these inventions would require different key word and sequence searches in different patent, non-patent literature and sequence databases and require, at least, specific searches for particular method steps of invention II not required for the search of invention I. These searches would then require subsequent in-depth analysis of all relevant prior art literature and sequence references, placing a serious burden on the Office in terms of both search and examination. As such, it would be burdensome to perform examination of inventions I and II together.

4. Because these inventions are distinct for the reasons given above, have acquired a separate status in the art as shown by their different classification and would require divergent searches of sequence and literature databases placing an undue administrative burden on the examiner, restriction for examination purposes as indicated is proper.

5. This application contains claims directed to the following patentably distinct species of the claimed invention: The diseases or conditions listed in claims 21-32 that are to be treated by the method of claim 20.

Applicant is required under 35 U.S.C. 121 to elect a single disclosed species for prosecution on the merits to which the claims shall be restricted if no generic claim is finally held to be allowable. Currently, claim 20 is generic.

Applicant is advised that a reply to this requirement must include an identification of the species that is elected consonant with this requirement, and a listing of all claims readable thereon, including any claims subsequently added. An argument that a claim is allowable or that all claims are generic is considered nonresponsive unless accompanied by an election.

Upon the allowance of a generic claim, applicant will be entitled to consideration of claims to additional species which are written in dependent form or otherwise include all the limitations of an allowed generic claim as provided by 37 CFR 1.141. If claims are added after the election, applicant must indicate which are readable upon the elected species. MPEP § 809.02(a).

Should applicant traverse on the ground that the species are not patentably distinct, applicant should submit evidence or identify such evidence now of record showing the species to be obvious variants or clearly admit on the record that this is the case. In either instance, if the examiner finds one of the inventions unpatentable over the prior art, the evidence or admission may be used in a rejection under 35 U.S.C. 103(a) of the other invention.

Status of the Application

6. During a telephone conversation with Christopher Bauer on 10/15/04 a provisional election was made with traverse to prosecute the invention of Group II, claims 19-32 and the species of disease/condition that is diabetes as set forth in claim 28. Affirmation of this election must be made by applicant in replying to this Office action. Claims 1-18, 21-27 and 29-32 are withdrawn from further consideration by the examiner, 37 CFR 1.142(b), as being drawn to a non-elected invention. It is noted herein that claims 19 and 20, which depend on withdrawn claim 2, are considered indefinite (see 112 2nd paragraph rejection below). However, in the interests of compact prosecution and because Applicant's phone election precluded amendment of the improper claim dependency, the limitations of claim 2 will be imparted into claims 19, 20 and 28 (which depends from claim 20) only insofar as the subject matter of claim 2 reads on the elected invention.

Priority

7. Applicant has not complied with one or more conditions for receiving the benefit of an earlier filing date under 35 U.S.C. 119(e) as follows: The instant application claims benefit of priority to an earlier filed application (U.S Provisional Application 60,419,268). However, support for claims 19, 20 and 28 (which depends from claim 20), drawn to methods of inhibiting mPGES-1, could not be found in the earlier filed document. If Applicant believes that such support is present in said document, Applicant should point out, with particularity, where such support is to be found.

Specification

8. The specification is objected to as failing to provide proper antecedent basis for the claimed subject matter. See 37 CFR 1.75(d)(1) and MPEP § 608.01(o). Correction of the following is required: Claims 19 and 20 (and claim 28 which depends from claim 20) are drawn to a method of treatment comprising inhibiting the expression of mPGES-1 in cells or tissues or a human using an antisense compound targeted to a nucleic acid molecule encoding a human GFAT. However, the meaning of the term “mPGES-1” is not apparent from the descriptive portion of the specification which does not provide any description of what “mPGES-1” is or refers to, or provide a clear disclosure as to its import.

Claim Rejections - 35 USC § 112

9. The following is a quotation of the second paragraph of 35 U.S.C. 112:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

10. Claims 19 and 20 (and claim 28 which depends from claim 20) are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Claims 19 and 20 recite the limitation "the antisense compound of claim 2". There is insufficient antecedent basis for this limitation in these claims because claim 2 has been withdrawn in the instant application. Additionally, claims 19 and 20 are both drawn to methods of inhibiting the expression of “mPGES-1.” However, one of skill in the art cannot determine the metes and bounds of claims drawn to methods of inhibiting

the expression of mPGES-1 because the nature or identity of mPGES-1 cannot be determined (without making assumptions as to what is represented by the abbreviation "mPGES-1) from the disclosures of either the specification or the claims as filed. What is mPGES-1?

11. The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

12. Claims 19, 20 and 28 (which depends from claim 20) are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. This is a written description rejection.

Claims 19 and 20 (and claim 28 which depends from claim 20) are drawn to a method of treatment as outlined previously in this action. The instant claims are drawn to a method of treatment wherein an antisense compound targeted to the nucleotide sequence encoding GFAT is used to inhibit the expression of mPGES-1.

The specification as filed, however, does not disclose or describe anything in regards to mPGES-1, providing no specific or general guidance as to what is encompassed by the method of treatment as claimed. Therefore, the claim(s) contains subject matter which was not described in the specification in such a way as to

reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

Vas-Cath Inc. v. Mahurkar, 19USPQ2nd 1111, clearly states that "applicant must convey with reasonable clarity to those skilled in the art that, as of the filing date sought, he or she was in possession of the invention. The invention is, for purposes of the "written description" inquiry, whatever is now claimed (see page 1117). Whether the specification shows that applicant was in possession of the claimed invention is not a single, simple determination, but rather is a factual determination reached by considering a number of factors. Factors to be considered in determining whether there is sufficient evidence of possession include the level of skill and knowledge in the art, partial structure, physical and/or chemical properties, functional characteristics alone or coupled with a known or disclosed correlation between structure and function, and the method of making the claimed invention.

Therefore, because there is no disclosure of any kind concerning mPGES-1 in the specification as filed, there is no indication that applicant was in possession of a method of treatment comprising inhibiting mPGES-1 using an antisense compound targeted to GFAT.

13. Claims 19, 20 and 28 (which depends from claim 20) are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the enablement requirement. The claim(s) contains subject matter which was not described in the specification in such a

way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

The invention of claims 19, 20 and 28 is outlined above. All claims are drawn to a method of treatment comprising inhibiting the expression of mPGES-1 using an antisense compound targeted to GFAT. In the instant case, the specification does not enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the method as claimed.

The following factors as enumerated *In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988), are considered when making a determination that a disclosure is not enabling: the breadth of the claims, the nature of the invention, the state of the prior art, the level of ordinary skill in the art, the level of predictability in the art, the amount of direction provided by the inventor, the existence of working examples and the quantity of experimentation needed to make the invention based on the content of the disclosure.

The scope of claims 19, 20 and 28 is broadly drawn to a method of treatment comprising inhibiting the *in vivo* expression of mPGES-1 in any cells or tissues of any organism (including humans). The specification as filed, however, provides no support for claims to a therapeutic method of inhibiting mPGES-1 using an antisense compound targeted to GFAT or, in fact, any other antisense compound because the specification provides no disclosure of any kind concerning the indentity or nature of mPGES-1. It is respectfully pointed out here that it is unclear how the skilled artisan would practice the method of inhibiting the expression of mPGES-1 using an antisense compound targeted

to GFAT when the identity or nature of mPGES-1 is unknown and cannot be determined (as set forth above in the rejection under 112 2^{nd} paragraph).

The methods recited in these claims indicate that the nature of this invention is a technique for antisense inhibition of gene expression *in vivo*, specifically an *in vivo* therapeutic method of antisense inhibition of mPGES-1 gene expression in cells, tissues or organisms using an antisense compound targeted to GFAT. The specification provides no disclosure of the identity of mPGES-1, no disclosure of methods of inhibiting the expression of mPGES-1, no examples of antisense inhibition of mPGES-1 gene expression using antisense compounds targeted to GFAT and no specific or general guidance that would allow the skilled artisan to practice the method as claimed. The specification as filed fails to even mention mPGES-1.

The state of the art at the time of filing, relative to the enablement of the antisense therapies *in vivo,* recognizes that there is a high degree of unpredictability in the art due to obstacles that continue, to the present day, to hinder the therapeutic application of nucleic acids *in vivo* (whole organism) including for example, problems with delivery and target accessibility. The following references discuss the problems of nucleic acid based therapies in reference to the claimed therapeutic antisense method.

Opalinska et al. 2002 (Nature Reviews, Vol. 1, pp. 503-514) provide a review of the challenges that remain before nucleic acid therapy becomes routine in therapeutic settings and clearly indicate that the art of nucleic acid therapy remains highly unpredictable and unreliable, particularly *in vivo*. According to Opalinska et al., "Although conceptually elegant, the prospect of using nucleic acid molecules for treating

human malignancies and other diseases remains tantalizing, but uncertain. The main cause of this uncertainty is the apparent randomness with which these materials modulate the expression of their intended targets. It is a widely held view that molecule delivery, and selection of which messenger RNA sequence to physically target, are core stumbling blocks that hold up progress in the field" (pg 503). Opalinska et al. also note that .. "[I]t is widely appreciated that the ability of nucleic acid molecules to modify gene expression *in vivo* is quite variable and therefore wanting in terms of reliability. Several issues have been implicated as a root cause of this problem, including molecule delivery to targeted cells and specific compartments within cells, and identification of sequence that is accessible to hybridization in the genomic DNA or RNA" (pg. 511).

In regards to the delivery of therapeutic nucleic acids, Jen et al. (Stem Cells 2000, Vol. 18, p 307-319) state (pg. 313, second column, second paragraph) "One of the major limitations for the therapeutic use of AS-ODNS and ribozymes is the problem of delivery.... presently, some success has been achieved in tissue culture, but efficient delivery for *in vivo* animal studies remains questionable". Jen et al. outlines many of the factors limiting the application of antisense for therapeutic purposes and concludes (pg. 315, second column), "Given the state of the art, it is perhaps not surprising that effective and efficient clinical translation of the antisense strategy has proven elusive."

Crooke, 2004 (Annu. Rev. Med. Vol. 55, pp. 61-95) discusses the particular problems associated with target accessibility wherein he states (in post filing art), "Selection of sites for induction of optimal antisense activity in an RNA molecule is dependent on the terminating mechanism and influenced by the chemical class of the

compound. Each RNA appears to display unique patterns of sites of sensitivity. Within the phosphorothiopate oligodeoxynucleotide chemical class, antisense activity can vary from undetectable to 100% by shifting an compound by just a few bases in the RNA target (references omitted). Despite significant progress in developing general rules that help define potentially optimal sites in RNA species, to a large extent, this remains an empirical process that must be performed for each RNA target and every new chemical class of compounds" (pg. 71, 4th paragraph).

Given this unpredictability, in particular in regards to targeting and delivery of antisense compounds, the skilled artisan would require specific guidance to practice the claimed method *in vivo*, with a resultant therapeutic outcome, as claimed. The instant specification does not show how one in the art might overcome the obstacles to providing antisense therapy as outlined above or how applicant has overcome the same general obstacles to antisense therapy in the instant invention.

Additionally, because no specific or functional species of antisense compounds that would be necessary to practice the method of treatment as claimed are disclosed in the specification, the skilled artisan would have to perform an extremely large and undue quantity of trial and error experimentation (as indicated above) in order to determine *de novo* the structure and function of an antisense compound that would function in the *in vivo* method of treatment as claimed. Based on the complete lack of guidance in the specification regarding the direction in which the experimentation should proceed, even if the *de novo* experiments required were considered routine by those of skill in the art, the more or less standard nature of each experiment would be

outweighed by the sheer quantity of undue trial and error experimentation required to determine how to practice the method of the instant invention.

In conclusion, due to the nature of the invention as an *in vivo* method of treatment, the degree of unpredictability in the art of antisense therapy, the breadth of the claimed method as an *in vivo* method of treatment for diabetes in any cells, tissues or organism, the lack of guidance as to what particular species of antisense nucleic acids would be required to practice the method as claimed, the need to screen multiple species of said nucleic acids so as to allow identification of particular species as functional within the method of treatment as claimed and the quantity of *de novo* experimentation necessary to discover the above, an undue amount of experimentation would be required in order to practice the method of treatment as claimed. Therefore, the inventors have not enabled one skilled in the art perform the method of the claimed invention.

Claim Rejections - 35 USC § 101

14. Claims 19, 20 and 28 are rejected under 35 U.S.C. 101 because the claimed invention is not supported by either a specific and substantial asserted utility or a well established utility. Claims 19 and 20 (and claim 28 which depends from claim 20) are drawn to a method of treatment as outlined previously in this action. However, the instant specification does not disclose or provide any suggestion or indication as to what is being claimed by a method of inhibiting “mPGES-1” because the specification does

not disclose or provide any suggestion or indication as to what “mPGES-1” is. Therefore, claims drawn to a method of inhibiting “mPGES-1” are not supported by a specific and substantial asserted utility (because none has been set forth in the instant specification) or a well established utility (because the nature or identity of “mPGES-1” cannot be determined).

Claims 19, 20 and 28 are also rejected under 35 U.S.C. 112, first paragraph. Specifically, since the claimed invention is not supported by either a specifc and substantial asserted utility or a well established utility for the reasons set forth above, one skilled in the art clearly would not know how to use the claimed invention.

Conclusion

15. No claim currently under examination is in condition for allowance. Claims 19, 20 and 28 were free of the prior art searched.

16. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Jon B. Ashen whose telephone number is 571-272-2913. The examiner can normally be reached on 7:30 am - 4:30 pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, John LeGuyader can be reached on 571-272-0670. The fax phone number for the organization where this application or proceeding is assigned is 703-872-9306.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to (571) 272-0547.

Patent applicants with problems or questions regarding electronic images that can be viewed in the Patent Application Information Retrieval system (PAIR) can now contact the USPTO's Patent Electronic Business Center (Patent EBC) for assistance. Representatives are available to answer your questions daily from 6 am to midnight (EST). The toll free number is (866) 217-9197. When calling please have your application serial or patent number, the type of document you are having an image problem with, the number of pages and the specific nature of the problem. The Patent Electronic Business Center will notify applicants of the resolution of the problem within 5-7 business days. Applicants can also check PAIR to confirm that the problem has been corrected. The USPTO's Patent Electronic Business Center is a complete service center supporting all patent business on the Internet. The USPTO's PAIR system provides Internet-based access to patent application status and history information. It also enables applicants to view the scanned images of their own application file folder(s) as well as general patent information available to the public. For all other customer support, please call the USPTO Call Center (UCC) at 800-786-9199.

Jba

SEAN McGARRY
PRIMARY EXAMINER
1635

Notice of References Cited	Application/Control No. 10/688,706	Applicant(s)/Patent Under Reexamination BROSCHAT ET AL.	
	Examiner Jon B. Ashen	Art Unit 1635	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Opalinska et al. Nucleic acid therapeutics: Basic principles and recent applications. Nature Review, 2002, vol 1, pp. 503-514
	V	Jen et al. Suppression of gene expression by targeted disruption of messenger RNA: available options and current strategies. Stem Cells 2000, Vol. 18, pp. 307-319)
	W	Crooke, S.T. Progress in antisense technology 2004 Annu. Rev. Med. Vol. 55, pp. 61-95
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

NUCLEIC-ACID THERAPEUTICS: BASIC PRINCIPLES AND RECENT APPLICATIONS

Joanna B. Opalinska and Alan M. Gewirtz‡*

The sequencing of the human genome and the elucidation of many molecular pathways that are important in disease have provided unprecedented opportunities for the development of new therapeutics. The types of molecule in development are increasingly varied, and include antisense oligonucleotides and ribozymes. Antisense technology and catalytic nucleic-acid enzymes are important tools for blocking the expression of abnormal genes. One FDA-approved antisense drug is already in the clinic for the treatment of cytomegalovirus retinitis, and other nucleic-acid therapies are undergoing clinical trials. This article reviews different strategies for modulating gene expression, and discusses the successes and problems that are associated with this type of therapy.

EXOGENOUS NUCLEIC ACIDS
In this context, synthetic oligonucleotides of varying chemistry (typically 16–25 nucleotides), which are introduced into cells by various means, or simply (although inefficiently) by concentration-driven endocytosis.

ANTISENSE
Reverse complement of any DNA or RNA sequence.

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With their promise of high specificity and low toxicity, many believe that gene-targeted therapies will lead to a revolution in cancer therapeutics[1]. Numerous gene-therapy strategies are under development, some of which use nucleic-acid-based molecules to inhibit gene expression at either the transcriptional or post-transcriptional level[2]. This strategy clearly has other potential applications, including in cardiovascular[3,4], inflammatory[5,6] and infectious diseases[7–10], as well as organ transplantation[11].

Although conceptually elegant, the prospect of using nucleic-acid molecules for treating human malignancies and other diseases remains tantalizing, but uncertain[12]. The main cause of this uncertainty is the apparent randomness with which these materials modulate the expression of their intended targets. It is a widely held view that molecule delivery, and selection of which messenger RNA sequence to physically target, are core stumbling blocks that hold up progress in the field. In this review, we recapitulate the development of nucleic-acid drugs for modulating gene expression, discuss newer strategies for solving the problems alluded to above, and detail attempts at using these molecules therapeutically. In so doing, we hope to both educate the reader who is unfamiliar with this literature, and convince those who are sceptical that this remains a viable approach to 'on demand' manipulation of gene expression.

Modulating gene expression

The notion that gene expression could be modified through the use of EXOGENOUS NUCLEIC ACIDS derives from studies by Paterson *et al.*[13], who first used single-stranded DNA to inhibit translation of a complementary RNA in a cell-free system in 1977. The following year, Zamecnik and Stephenson[14] showed that a short (13-mer) DNA oligodeoxynucleotide that was ANTISENSE to the Rous sarcoma virus could inhibit viral replication in culture. On the basis of this work, Zamecnik and Stephenson are widely credited for having first suggested the therapeutic utility of antisense nucleic acids. In the mid 1980s, the existence of naturally occurring antisense RNAs and their role in regulating gene expression was shown[15,16]. These observations were particularly important, because they lent credibility to the belief that 'antisense' was more than just a laboratory phenomenon, and encouraged belief in the hypothesis that reverse-complementary antisense nucleic acids could be used in living cells to manipulate gene expression. These seminal papers, and the thousands that have followed, have stimulated the development of technologies that use nucleic acids to manipulate gene expression. As will be discussed below, virtually all of the available methods rely on some type of nucleotide-sequence recognition for targeting

TRIPLE-HELIX-FORMING OLIGODEOXYNUCLEOTIDE (TFO). A synthetic, single-stranded oligodeoxynucleotide, which, through Hoogsten-bond formation, hybridizes to purine/pyrymidine-rich sequences in double-stranded DNA. Formation of stable triple helices can prevent the unwinding that is necessary for transcription of the targeted region or block the binding of transcription-factor complexes.

MAJOR GROOVE AND MINOR GROOVE
Channels formed by the twisting of two complementary DNA strands around each other to form a double helix. The major groove is ~22 Å wide and the minor groove is ~12 Å wide.

HOOGSTEEN BOND
Triple-helix-forming oligonucleotides hybridize with purine bases that comprise polypurine/polypyrimidine tracks in the DNA. The hydrogen bonds that are formed under these conditions are referred to as Hoogsteen bonds after the individual who first described them. They can form in parallel or antiparallel (reverse-Hoogsteen) orientations.

NUCLEOSOME
A packing unit for DNA within the cell nucleus, which gives the chromatin a 'beads-on-a-string' structure. The 'beads' consist of complexes of nuclear proteins (histones) and DNA, and the 'string' consists of DNA only. A histone octamer forms a core around which the double-stranded DNA helix is wound twice.

LEXITROPSIN
A molecule that extragenetically reads the base sequence of double-stranded DNA.

RIBOZYME
RNA molecule that contains one of a variety of catalytic motifs that cleave RNA to which it hybridizes.

DNAzyme
A DNA molecule that contains a catalytic motif that cleaves RNA to which it hybridizes.

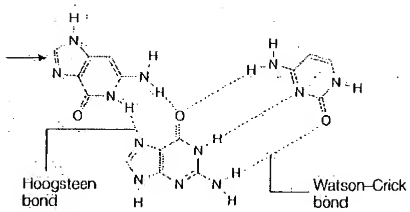

Figure 1 | **Triple-helix formation at the nucleotide level.** Shows the formation of Watson–Crick (red) and Hoogsteen bonds (black) between duplex pairs and the third strand (the arrow points to a single base of the third strand). Blue, guanine residue (purine); pink, cytosine residue (pyrimidine).

specificity, but differ as to where and how they perturb the flow of genetic information.

Strategies for modulating gene expression can be thought of as being either 'anti-gene' or anti-mRNA (see below; reviewed in REF. 2). Anti-gene strategies focus primarily on gene targeting by homologous recombination[17,18], or by TRIPLE-HELIX-FORMING OLIGODEOXYNUCLEOTIDES (TFOs)[19]. As homologous recombination involves vector technology and — at least at the present time — is much too inefficient for clinical use, it will not be considered further in this discussion. TFOs bind in the MAJOR GROOVE of duplex DNA in a sequence-specific manner[20]. Gene targeting with these molecules is constrained by the fact that TFOs require runs of purines on one strand and pyrimidines on the other (~10–30 nucleotides (nts) in length) for stable hybridization. The TFO can be composed of either polypurine or polypyrimidine tracts, but hybridization always occurs on the purine strand of the duplex through the formation of HOOGSTEEN BONDS (FIG. 1).

Successful use of this strategy for blocking transcription and inducing specific mutations, both *in vitro* and *in vivo*, has been reported (reviewed in REF. 20). Although the frequency of such events is typically <1%, Glazer and co-workers[21] have reported a system in which desired mutations can be induced in ~50% of cells, indicating that genuine clinical utility might be possible. This general approach has also been used for inducing mutations that can actually repair a gene that has been made defective by inherited or acquired point mutation. Work to support this concept using chimeric DNA–RNA oligonucleotides has also been reported, but again, the frequency of such repairs, in most cases, has been far too low to be of clinical use at this time[22].

Short, double-stranded (ds)DNA decoy molecules have also been used to disrupt gene expression at the level of transcription[23]. These oligodeoxynucleotides are designed to compete for transcription-factor complexes, with the ultimate goal of attracting them away from the promoter that they would ordinarily activate. For many technical reasons, including limited gene accessibility in the NUCLEOSOME structure, the clinical application of these methods has not progressed at a rapid rate. An alternative approach, using polyamides, or LEXITROPSINS, has been described by Dervan and colleagues[24–26]. These small molecules have the ability to diffuse into the nucleus, where they can contact dsDNA in the minor groove, thereby impeding transcription by preventing unwinding of the duplex, or by preventing the binding of transcription-factor complexes to the gene promoter. DNA accessibility, and maintaining the appropriate 'register' of the polyamides for the desired sequence recognition, are problems with this method that remain to be solved[27].

A larger body of work has focused on destabilizing mRNA. This approach, although less favourable than anti-gene strategies from a stoichiometric point of view, is nonetheless attractive, because mRNA, unlike the DNA of a given gene, is — theoretically— accessible to attack while being transcribed, transported from the nucleus or translated. Two nucleic-acid-based strategies have emerged for blocking translation. One strategy uses oligoribonucleotides. Similar to the strategy of the DNA decoys, the RNA decoys are designed to provide alternate, competing binding sites for proteins that act as translational activators or mRNA-stabilizing elements[28,29]. By attracting away the desired protein, the decoy can prevent translation, or induce instability and, ultimately, destruction of the mRNA. Recent studies on human α-globin mRNA are of interest in this regard. Stability determinants for this mRNA species have been defined in sufficient detail so that it can be used as a model system for testing the hypothesis that altering mRNA stability with decoys will be a useful form of therapy[29–31].

The other strategy for destabilizing mRNA is the more widely applied antisense strategy, which uses RIBOZYMES, DNAzymes, antisense RNA or antisense DNA (ODN). The antisense approach to modulating gene expression has been the subject of numerous authoritative reviews, and will not be discussed in great detail here[2,32,33]. Simply stated, delivering a reverse-complementary — that is, 'antisense' — nucleic acid into a cell in which the gene of interest is expressed should lead to hybridization between the antisense sequence and the mRNA of the targeted gene. Stable mRNA–antisense duplexes can interfere with the splicing of heteronuclear RNA into mature mRNA[34,35], block translation of completed message[36,37] and — depending on the chemical composition of the antisense molecule —lead to the destruction of the mRNA by binding of endogenous nucleases, such as RNaseH[38,39], or by intrinsic enzymatic activity engineered into the sequence, as is the case with ribozymes[40,41] and DNAzymes[42,43] (FIG. 2).

Nucleic acids with catalytic activity

Ribozymes and DNAzymes bind to substrate RNA through Watson–Crick base pairing, which offers sequence-specific cleavage of transcripts. At least six classes of ribozyme have been described. Two ribozymes, the 'hammerhead' ribozyme and the 'hairpin' ribozyme, have been extensively studied owing to their small size and rapid kinetics[44,45]. The catalytic motif is surrounded by flanking sequence that is responsible for 'guiding' the ribozyme to its mRNA target and giving stability to the structure. With the hammerhead ribozyme, cleavage is dependent on divalent cations, such as magnesium, and can occur after any NUH

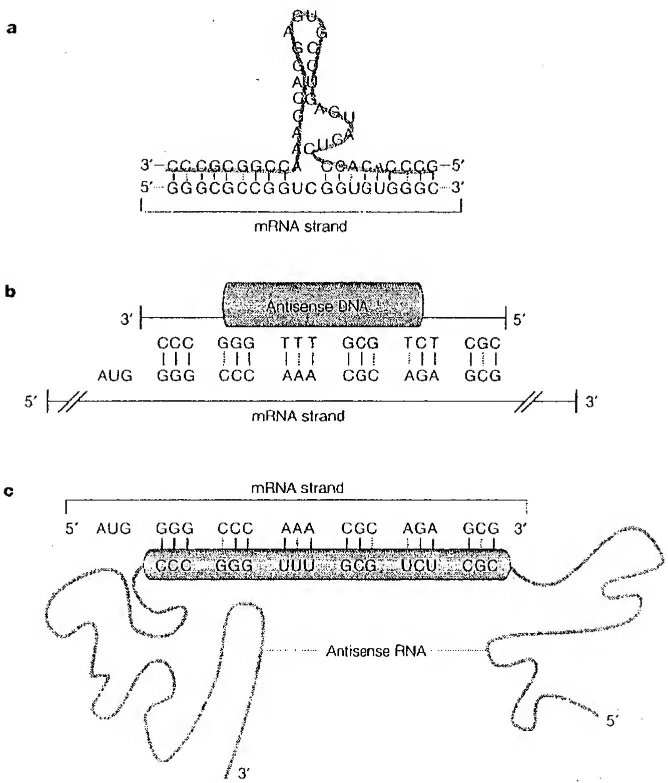

Figure 2 | **Strategies for inhibiting translation.** Diagrammatic representations of **a** | a hammerhead ribozyme (DNAzymes have similar RNA-cleaving capabilities, but the catalytic motif is composed of DNA nucleotides, hence the name); **b** | an antisense oligodeoxynucleotide; and **c** | antisense RNA. Note that targeting specificity is conveyed in each case by Watson–Crick base pairing between complementary sequences. From REF. 2 © (1998) American Society of Hematology, used by permission. mRNA, messenger RNA.

triplet within the target RNA sequence, for which 'N' represents any nucleotide, 'U' represents uracil and 'H' represents adenine, cytosine or uracil[46,47]. If ribozymes are to work effectively as 'enzymes,' they must not only bind substrate RNA but also dissociate from the cleavage product to act on further substrates. Dissociation from the cleavage product might, in fact, be an important rate-limiting step that controls their usefulness[48,49]. Consideration of reaction kinetics indicates that ribozymes might have a theoretical advantage over RNase-H-dependent antisense oligonucleotides, but to the best of our knowledge, this has not been shown consistently *in vivo*. Ribozymes can be expressed from a vector that offers the advantage of continued production of these molecules intracellularly[50,51], a property that — at least until recently — was not possible with antisense DNA[52]. However, it is well known that stable transduction of primary cells *in vivo* has substantial technical problems, which will not be discussed further. Progress has been made recently in synthesizing stable forms of these molecules, so that they might be delivered directly to cells both *in vitro* and *in vivo*[53].

DNAzymes have evolved from the seminal work of Breaker and Joyce[54], who first showed that DNA, as well as RNA molecules, could act enzymatically and cleave a nucleic-acid substrate. Similar to ribozymes, DNAzymes have a catalytic domain that is flanked by two substrate-recognition domains. After binding to their RNA substrate, DNAzymes can cleave sequences that contain purine–pyrimidine junctions. DNAzymes have some theoretical advantages over ribozymes. DNA is more stable than RNA, it is easier to synthesize, and the turnover rates for some of the DNAzymes are reported to be higher than some ribozymes[42]. Nevertheless, constant improvements in both DNAzyme[55] and ribozyme chemistry make this a 'moving target' in terms of which chemistry is better[56]. Although experience with DNAzymes as potential therapeutic agents is limited[43], these molecules might prove worthy in the clinical setting.

RNA interference

A newly developing approach for targeting mRNA is called post-transcriptional gene silencing, or RNA interference (RNAi)[57–58] (FIG. 3). RNAi is the process by which dsRNA targets mRNA for destruction in a sequence-dependent manner. The mechanism of RNAi initially involves processing of long (~500–1,000 nucleotides) dsRNA into 21–25 base-pair (bp) 'trigger' fragments[59] by a member of the RNase-III family of nucleases called DICER[60–62]. When incorporated into a larger, multicomponent nuclease complex named RISC (RNA-induced silencing complex), the processed trigger strands form a 'guide sequence' that targets the RISC to the desired mRNA sequence and promotes its destruction[61]. RNAi has been used successfully for gene silencing in various experimental systems, including petunias, tobacco plants, neurospora, *Caenorhabditis elegans*, insects, planaria, hydra and zebrafish. The use of long dsRNA to silence expression in mammalian cells has been tried, largely without success[63]. More recent reports using short interfering RNA (siRNA; see below) seem to be more promising[64]. It has been suggested that mature, as opposed to embryonic, mammalian cells recognize these long dsRNA sequences as invading pathogens. This triggers a complex host-defence reaction that effectively shuts down all protein synthesis in the cell through an interferon-inducible serine/threonine-kinase enzyme called protein kinase R (PKR). PKR phosphorylates the α-subunit of eukaryotic initiation factor-2 (EIF-2α), which globally inhibits mRNA translation. The long dsRNA also activates 2′,5′-oligoadenylate synthetase, which in turn activates RNase L. RNase L indiscriminately cleaves mRNA. Cell death is the understandable result of these processes. Recently, a number of reports have suggested that siRNA strands — RNA double strands of ~21–22 nucleotides in length — do not trigger this host-defence response, and therefore might be able to silence expression in mammalian somatic cells if appropriately modified to contain 3′-hydroxy and 5′-phosphate groups[66–68]. The universality of this approach, and the types of gene that can be modified using this strategy in mammalian cells, remain unknown at this time.

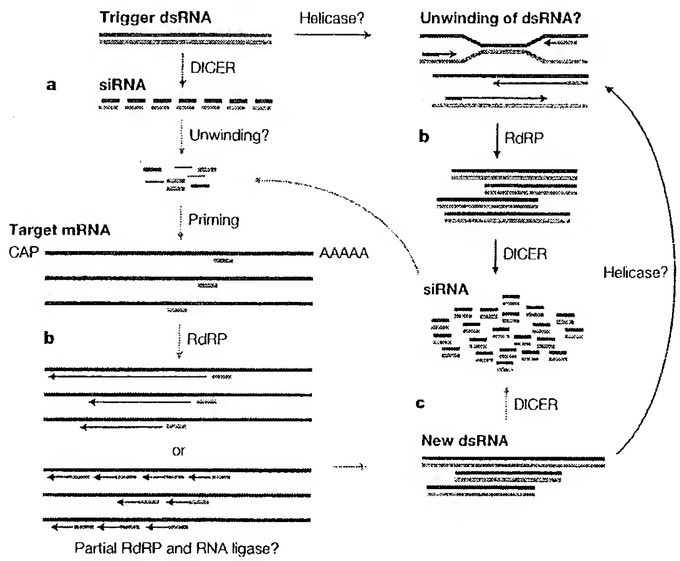

Figure 3 | **Hypothetical RNAi mechanism. a** | In the 'initiation' stage of RNA interference (RNAi), a small amount of trigger double-stranded (ds)RNA is processed into short interfering (si)RNA by an enzyme called DICER (light blue arrow), which is used as an RdRP primer. **b** | The RdRP reaction converts target messenger RNAs into new dsRNAs (next generation of trigger dsRNAs), which are then processed into new siRNAs, establishing a self-sustaining cycle of RNAi 'maintenance' (green arrows). **c** | Replication of 'trigger' or newly synthesized dsRNA by RdRP would amplify the potency of RNAi by further increasing the amount of siRNA, as both sense and antisense strands of trigger dsRNA and siRNA could then be used. However, the *in vivo* significance of this pathway (dark grey arrows) has not yet been established. It also remains unclear if the 'amplification' steps take place in mammalian cells. RdRP, RNA-dependent RNA polymerase; helicase, unwinding enzyme. Redrawn from REF. 57 © (2001), with permission from Elsevier Science.

Altering RNA splicing

Finally, the strategy of manipulating gene expression by altering RNA processing, as opposed to by mRNA destruction, is also worth mentioning, as significant progress seems to have been made in this area. Kole and colleagues developed this approach using a model system based on human thalassaemia[69,70]. Thalassaemias are highly prevalent human blood disorders that are characterized by faulty haemoglobin production and concomitant red-cell destruction that results in anaemia. The genetic mutations that are responsible for these diseases are well characterized, and often involve aberrant splicing. Kole's group showed that treatment of mammalian cells that were stably expressing a human β-globin gene with antisense oligonucleotides that were targeted at the aberrant splice sites blocked the abnormal splicing, thereby allowing the normal splice site to be used. Correction of splicing was oligo-dose dependent and, importantly, led to accumulation of normal human β-globin mRNA and polypeptide in cells[69]. More recently, correction has been accomplished in blood cells derived from thalassaemic patients[71]. This result would clearly have important clinical consequences if such treatment could be made effective at the level of the haematopoietic stem cell. These same workers suggest that this approach might also be useful in the treatment of cancer[72].

Increasing oligonucleotide stability

Initial work with antisense DNA was carried out with unmodified, natural molecules. It soon became clear, however, that native DNA was subject to relatively rapid degradation, primarily through the action of 3' exonucleases, but also as a result of endonuclease attack. Molecules destined for the clinic, and those used for experimental purposes, are now routinely modified to enhance their stability, as well as the strength of their hybridization with RNA (see REFS 73,74 for further details). Oligonucleotide drugs need to meet certain physical requirements to make them useful. First, they must be able to cross cell membranes and then hybridize with their intended target. The ability of an ODN to form a stable hybrid is a function of its binding affinity and sequence specificity. Binding affinity is a function of the number of hydrogen bonds that are formed between the ODN and the sequence to which it is targeted. This is measured objectively by determining the temperature at which 50% of the double-stranded material is dissociated into single strands, which is known as the melting temperature, or T_m. mRNA-associated proteins and tertiary structure also govern the ability of an ODN to hybridize with its target by physically blocking access to the region that is being targeted by the ODN. Finally, it is also clear that ODNs should exert little in the way of non-sequence-related toxicity[75], and should remain stable in the extracellular and intracellular milieu in which they are situated. Meeting all these requirements in any one molecule has turned out to be a demanding task. Satisfying one criterion is often accomplished at the expense of another. It is also worth noting that the more complex the molecule, the more expensive is its synthesis. In an age of increasing cost consciousness, this too becomes an important design consideration.

First-generation antisense molecules were designed to make the internucleotide linkages — the backbone on which the nucleosides are hung — more resistant to nuclease attack. This was accomplished primarily by replacing one of the non-bridging oxygen atoms in the phosphate group with either a sulphur or a methyl group. The former modification, which is called a phosphorothioate oligodeoxynucleotide, proved highly successful, because these molecules are relatively nuclease resistant, they are charged and therefore water soluble, and they activate RNase H. All of these properties are desirable, and virtually all of the clinical trials done so far have been carried out with this chemistry, although trials using so-called 'second-generation molecules' (mixed backbone/chimeric oligonucleotides) will shortly begin. Second-generation molecules were developed to overcome the disadvantageous properties of the phosphorothioates. A primary strategy that was used was to remove the phosphorothioate linkages to the greatest extent possible. This was often done by flanking a phosphorothioate core with nuclease-resistant nucleosides — often with 2'-O sugar modifications — that rendered the molecules more RNA like, and therefore gave tighter binding to the target.

Many chemical modifications to the phosphodiester linkage have been made. Two of the more interesting modifications that are now under development are peptide nucleic acids (PNAs)[76] and MORPHOLINO OLIGODEOXYNUCLEOTIDES (PMOs)[36]. These compounds are essentially nuclease resistant. PNAs represent a more radical approach to the nuclease-resistance problem, as the phosphodiester linkage is completely replaced with a polyamide (peptide) backbone. They both form extremely tight bonds with their RNA targets and probably exert their effects by blocking translation, as neither molecule effectively activates RNase H. Whether it is necessary to preserve the ability of these molecules to activate RNase H is controversial[37], but many workers in the field still believe that molecules with this capability are likely to be more effective, at least in the clinical setting. As these molecules do not move freely across cell membranes, they must be injected or transfected into cells. Finally, PNAs are also sensitive to local ionic concentration and do not hybridize as well under physiological conditions.

Nucleic-acid drugs in the clinic

Diseases that are characterized by overexpression or inappropriate expression of specific genes, or genes that are expressed by invading microorganisms, are candidates for gene-silencing therapies. For this reason, the earliest clinical trials with these agents have been against human immunodeficiency virus (HIV)77–79 and patients with cancer80. Malignant diseases, in particular, are attractive candidates for this therapeutic approach, if for no other reason than that conventional cancer therapies are highly toxic. As antisense strategies are directed against genes that are aberrantly expressed in diseased cells, it might reasonably be expected that this approach will engender fewer and less serious side effects, as normal cells should not be affected. There were concerns that this might not be the case when preclinical studies on primates with phosphorothioate compounds resulted in the death of some animals. However, investigation of these occurrences showed that they took place after rapid bolus intravenous infusions at concentrations exceeding 5–10 μg ml^{-1}, and that they were probably due to complement activation and vascular collapse[81].

MORPHOLINO OLIGODEOXYNUCLEOTIDE (PMO). The base is attached to a morpholino instead of a ribofuranosyl ring, and the backbone is composed of a phosphorodiamidate linkage.

This experience was therefore a useful reminder that, in addition to side effects resulting from the suppression of the targeted gene, side effects related to the chemical backbone of the oligonucleotide should also be anticipated. In the case of phosphorothioates, this problem was easily addressed by infusing material continuously, or slowly, and at lower doses. In actual use in the clinic, phosphorothioates have proved to be remarkably well tolerated (BOX 1). Abnormalities related to the backbone include transient fever, fatigue, nausea and vomiting, mild to moderate thrombocytopaenia and transient prolongation of partial thromboplastin time (PTT; 1.25–1.75 ×), which is fortunately unassociated with any signs of overt clinical bleeding[82–85]. At present, several clinical studies have been carried out using a number of different oligonucleotides. Below, we review some of the more recent clinical studies that have been carried out on patients with malignant, inflammatory, cardiac and infectious diseases (summarized in TABLE 1).

Targeting apoptosis inhibitors in oncology

BCL2: cancer treatment. Targeting B-cell lymphoma protein 2 (BCL2) is a promising example of triggering apoptosis in tumour cells. BCL2 is an important regulator of programmed cell death, and its overexpression has been implicated in the pathogenesis of some lymphomas[86]. Resistance to chemotherapy, at least *in vitro*, might also be related to *BCL2* overexpression[87,88]. Laboratory studies have shown convincingly that exposing cells to an oligonucleotide targeted to *BCL2* will specifically decrease the amount of targeted mRNA and protein (six–eightfold reduction). For all of these reasons, there is a great deal of interest in targeting *BCL2* for therapeutic purposes[89]. Several clinical trials with a *BCL2*-targeted antisense molecule have been reported, both alone[83,90] and with supplementary chemotherapy[84,91,92]. Studies with the oligonucleotide alone have not shown consistent, strong antitumour responses. The addition of chemotherapy might be helpful in this regard. An issue with several of these studies is lack of correlation of tumour responses with significant effects on BCL2 protein expression. The mechanism of action of the compound is not entirely clear.

Transcription-factor targeting in oncology

c-MYB: bone-marrow purging. The normal homologue of the avian myeloblastosis virus oncogene (v-*myb*) is a proto-oncogene called c-*MYB*. c-*MYB* encodes a protein (MYB), which is a regulator of cell-cycle transition and cellular maturation, primarily in haematopoietic cells, but in other cell types as well. A recently published study was designed to test the hypothesis that an effectively delivered, appropriately targeted ODN might provide a proof of concept about the ability to target a specific mRNA and thereby kill tumour cells selectively[93]. To test this hypothesis, an ODN targeted to the c-*MYB* proto-oncogene was used to purge marrow autografts that were administered to patients with allograft-ineligible chronic myelogenous leukaemia (CML). CD34$^+$ marrow cells were purged

Box 1 | First approved nucleic-acid drug

Vitravene (sodium fomivirsen), an antiviral drug that was developed by ISIS Pharmaceuticals and is marketed by CIBAVision, was approved by European and US regulatory authorities in July 1999 and August 1998, respectively. Vitravene is used to treat an inflammatory viral infection of the eye (retinitis) that is caused by the cytomegalovirus (CMV). CMV often infects immunocompromised patients, and patients with uncontrolled AIDS are particularly at risk. One or both eyes can be affected, and it is not unusual for patients to suffer severe visual impairment or blindness as a result of untreated infections. Treatment of CMV retinitis is problematic, in particular for patients who cannot take, do not respond or become resistant to standard antiviral treatments for CMV infections, such as ganciclovir, foscarnet and cidofovir[145]. Vitravene is an antisense phosphorothioate 21-mer oligonucleotide has a sequence that is complementary to messenger RNA that is transcribed from the main immediate–early transcriptional unit of CMV[145,146].

with ODN for either 24 ($n = 19$) or 72 ($n = 5$) hours (FIG. 4). Post-purging, c-*MYB* mRNA levels declined substantially in ~50% of patients. Analysis of *BCR–ABL* (breakpoint cluster region–Abelson murine leukaemia viral oncogene homologue) expression in a surrogate stem-cell assay indicated that purging had been accomplished at a primitive cell level in >50% of patients. Cytogenetics were evaluated at day 100 in surviving patients who did not require administration of unpurged 'rescue' marrow for engraftment ($n = 14$). (All purging protocols require storage of untreated marrow as a 'back-up', in case the purged material does not engraft.) Whereas all patients were ~100% Ph⁺ (Philadelphia chromosome positive) pre-transplant, two patients had complete cytogenetic remissions, three patients had <33% Ph⁺ metaphases and eight remained 100% Ph⁺. The marrow of one patient yielded no metaphases, but fluorescence *in situ* hybridization (FISH) evaluation ~18 months post-transplant revealed that ~45% of cells were *BCR–ABL*⁺, indicating that six

Table 1 | **Summary of recently published clinical trials with nucleic-acid drugs**

Target	Type of study	No. of patients	Diagnosis	Dose range	Treatment duration	Administration	Remissions	Refs
ICAM-1	Multicentre; placebo controlled; double blind	75	Crohn's disease	0.5 mg	2 days–4 weeks	SC	Not significant	106
	Placebo controlled; double blind	20	Crohn's disease	0.5–2 mg kg^{-1}	26 days	2 hours IV infusion	47% steroid-free remissions	6
PKC-α	Phase I	36	Advanced cancer	0.15–6 mg kg^{-1}d^{-1}	3 days per week for 3 weeks every 4 weeks	2 hours IV infusion	2 CR	82
	Phase I	21	Advanced cancer	0.5–3 mg kg^{-1}d^{-1}	21 days every 4 weeks	Continuous IV infusion	3 responses	85
BCL2	Phase I	21	Relapsed NHL	4.6–195.8 mg m^{-2} d^{-1}	14 days	Continuous SC infusion	1 CR, 2 minor responses	83
BCL2 combined with dacarbazine	Phase I/II	14	Advanced malignant melanoma	0.6-6.5 mg kg d^{-1}	14 days every 4 weeks	Continuous IV infusion	1 CR, 2 PR 3 minor responses	91
BCL2 combined with mitoxantrone	Phase I/II	26	Metastatic prostate cancer	0.6–5 mg kg^{-1} d^{-1}	14 days every 28 days	Continuous IV infusion	2 decreases in PSA	84
Fomivirsen CMV	Multicentre; randomized; prospective	29	CMV retinitis in AIDS patients	165 μg	Once per week	Intravitreously	Time to progression 71 versus 13 days	147
h-RAS	Phase I	23	Advanced cancer	0.5–10 mg kg^{-1} d^{-1}	14 days every 3 weeks	Continuous IV infusion	4 stable	96
c-RAF kinase	Phase I	34	Advanced cancer	1–5 mg kg^{-1} d^{-1}	21 days every 4 weeks	Continuous IV infusion	2 stable diseases	119
	Multicentre Phase II	22	SCLC and NSCLC	2 mg kg^{-1} d^{-1}	21 days every 4 weeks	Continuous IV infusion	No responses	148
	Phase I	22	Advanced cancer	6–30 mg kg^{-1} d^{-1}	Weekly	24 hours IV infusion	No responses	99
c-MYC	Multicentre; placebo controlled	78	After PTCA	1–24 mg d^{-1}	Single dose	Intracoronary	No responses	108
	Placebo controlled	85	After coronary-stent implantation	10 mg d^{-1}	Single dose	Intracoronary	No responses	109
IGF1R	Pilot study	12	Malignant astrocytoma	2 mg 10^{-7} cells	6 hours	*Ex vivo*	2 CR 6 PR	118

AS, antisense; BCL2, B-cell lymphoma protein 2; CMV, cytomegalovirus; CR, complete remission; ICAM-1, intercellular adhesion molecule-1; IGF1R, insulin-like-growth-factor-1 receptor; IV, intravenous; c-MYC, myelocytomatosis viral oncogene homologue; NHL, non-Hodgkin's lymphoma; NSCLC, non-small-cell lung cancer; PKC-α; protein kinase C-α; PR, partial remission; PSA, prostate-specific antigen; PTCA, percutaneous transluminal coronary angioplasty; SC, subcutaneous; SCLC, small-cell lung cancer.

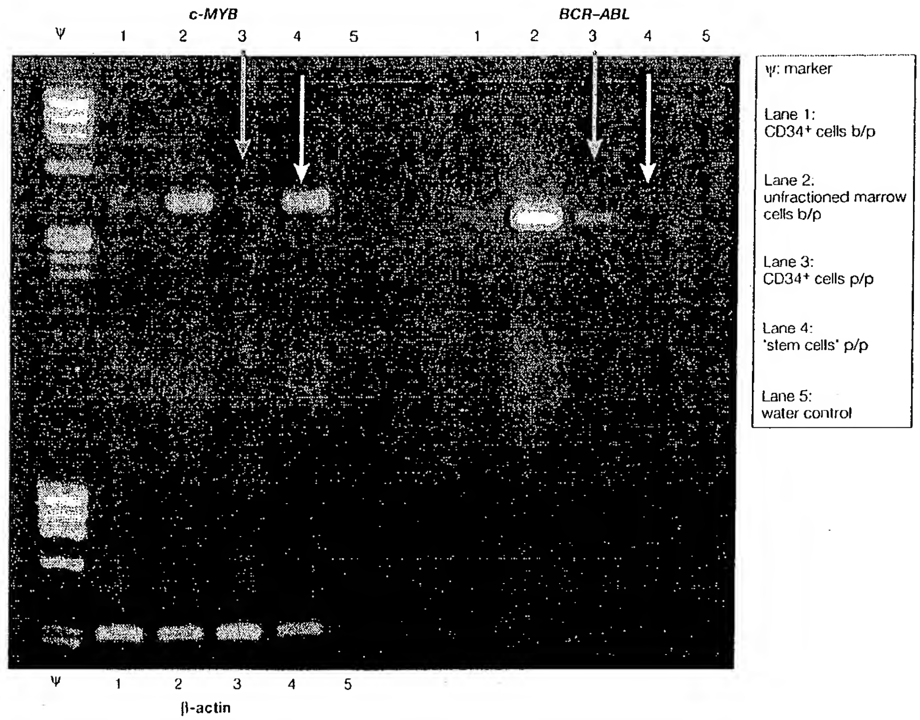

Figure 4 | **Effect of c-*MYB*-targeted ODNs on c-*MYB* mRNA expression in marrow cells.** Ethidium-bromide-stained agarose gel containing c-*MYB*, *BCR–ABL* and β-actin messenger RNA reverse transcriptase (RT)-PCR products derived from: $CD34^+$ bone-marrow cells of a representative patient before anti-c-*MYB* oligodeoxynucleotide purging (Lane 1); unfractionated bone-marrow cells before purging (Lane 2); $CD34^+$ cells post-purging (Lane 3); and the patient's primitive 'stem cells' post purging (Lane 4). A control RT-PCR reaction that contains only water is shown in Lane 5. Lanes containing molecular-weight markers are indicated by the symbol Ψ. Lane 3 (orange arrows) reveals that c-*MYB* mRNA is undetectable post purging, whereas some residual *BCR–ABL* expression (molecular marker of the malignant cells) persists. Efficiency of the process on primitive haematopoietic cells is shown in lane 4 (white arrows). Here, stem cells, cultured for ten days post-purge, show normal MYB expression, whereas *BCR–ABL* expression is undetectable. These data indicate that, in this patient's marrow sample, normal cells survived the purge but malignant, *BCR–ABL*-expressing cells did not. Control cells that were treated in an identical manner but not exposed to the anti-c-*MYB* oligodeoxynucleotide continue to express *BCR–ABL* (not shown), which indicates that the results are due to oligodeoxynucleotide exposure and are not a cell-culture artefact. b/p, before purging; p/p, post purging.

out of fourteen patients had originally obtained a 'major' cytogenetic response. Conclusions about clinical efficacy of ODN marrow purging could not be drawn from this small pilot study. Nevertheless, these results led the authors to speculate that enhanced delivery of ODN, targeted to crucial proteins with short half-lives, might lead to the development of more effective nucleic-acid drugs and enhanced clinical utility of these compounds in the future.

Oncogenic signal-transduction pathways

Protein kinase C-α. Protein kinase C (PKC) comprises a family of biochemically and functionally distinct phospholipid-dependent, cytoplasmic serine/threonine kinases. These proteins have a crucial role in transducing the signals that regulate cell proliferation and differentiation. PKC is overexpressed in several tumours, and antisense inhibitors of these enzymes have shown some antitumour activity *in vitro*[5,82,94] and in animal models[95]. Results of two studies that used the identical 20-mer phosphorothioate ODN against *PKCα* have been published[82,85]. The ODN was well tolerated, but antitumour effects were modest at best. Correlations with levels of *PKCα* expression were not provided.

RAS pathway

h-RAS oligonucleotide. h-RAS is a powerful regulator of several interconnected receptor-signalling pathways. The gene is constitutively active, and promotes proliferation and malignant transformation in many human tumours. Cunningham *et al.* reported results from a study that was carried out on 23 patients with various malignancies[96]. As in other studies with phosphorothioate oligonucleotides, only mild toxicities were observed. No complete or partial responses were achieved. Four patients had stabilized disease for 6–10 cycles of treatment.

c-RAF kinase. RAF proteins are crucial effectors in the RAS signal-transduction pathway. Constitutive activation of the RAS pathway is thought to contribute to malignant transformation in many cell types, which makes elements of this signalling pathway attractive targets for inhibition. Effectiveness of an antisense oligonucleotide against c-*RAF* has been shown both *in vitro*[97] and in an *in vivo* tumour-xenograft model[98]. On the basis of this work, three clinical trials were initiated[99,119,148]. A total of 78 patients were treated. No major tumour responses were documented, but some patients had stabilization of their disease.

Ribozymes

Ribozymes have been the subject of several authoritative reviews[41,100]. Although there is a comprehensive literature that describes the use of these molecules to target a wide variety of mRNA species in various cell-free, cell-intact and animal-model systems (see REFS 41,111), there is little recently published material on the use of these materials in clinical trials. The earliest clinical use of ribozymes was in patients with HIV[77,78,101,102]. As is true of antisense oligodeoxynucleotides, the approach was found to be safe when ribozymes were expressed in cells that were then delivered back to patients, but clinical efficacy was found wanting. At present, several Phase I/II clinical trials with exogenously delivered synthetic ribozymes are in early-phase clinical evaluation for patients with breast cancer, colon cancer and hepatitis. Results of these clinical investigations are anxiously awaited.

Studies in non-malignant diseases

Inflammatory diseases. Antisense oligonucleotides have been explored as anti-inflammatory agents. An example is the targeting of intracellular adhesion molecule-1 (ICAM-1) in Crohn's disease. In response to inflammatory stimuli, many cells upregulate the expression of *ICAM1*, which has an important role in the transport and activation of leukocytes. It has been shown *in vitro* and *in vivo* that administration of antisense oligonucleotides against *ICAM1* causes a decrease in receptor expression, which in turn ameliorates inflammatory reactions[103–105]. Two clinical trials with this compound in patients with Crohn's disease have been reported[6,106]. In the double-blind study reported by Yacyshyn *et al.*[6], 20 patients were randomized to receive a saline placebo or anti-*ICAM1* antisense oligonucleotide. The treatment was well tolerated, and after 6 months, disease remission was reported in 47% of patients in the antisense group compared with 20% of patients in the placebo group. Furthermore, corticosteroid usage was significantly lower ($p = 0.0001$) in the antisense-treated patients. These results engendered a great deal of excitement, but the enthusiasm was subsequently dampened by the follow-on study that was carried out with this compound in a larger group of patients with this disease ($n = 75$)[106]. In this placebo-controlled study, no statistically significant differences in steroid use between the treatment or placebo groups was observed, although 'positive trends' were seen in the patients who were treated with the antisense oligonucleotide. As with other studies, toxicity was mild and consisted primarily of pain at the injection site, fever and headache.

The anti-*ICAM1* oligonucleotide has also been evaluated in patients with psoriasis. The drug was initially administered by intravenous infusion to these individuals, but examination of their skin indicated that delivery to its various layers was poor. For this reason, a topical formulation was developed. Although preclinical data about uptake of this formulation into the skin and downregulation of expression of the target were encouraging[107], the ensuing clinical trial showed only modest, short-term effects in these patients (see the ISIS Pharmaceuticals web site online). The ultimate usefulness of this compound remains to be determined.

Cardiovascular disease. RESTENOSIS of coronary vessels after *trans*-catheter re-vascularization procedures remains a serious clinical problem. Manipulation of coronary vessels invariably leads to endothelial-cell injury, which is often accompanied by thrombosis, smooth-muscle-cell activation and subsequent vascular remodelling. The myelocytomatosis viral oncogene homologue (c-MYC) has been identified as an important mediator in this process through its effects on regulating the growth of vascular cells in atherosclerotic lesions. Accordingly, it has been postulated that c-*MYC* might make an attractive target for preventing post-angioplasty complications, and at least two clinical trials using a 15-mer phosphorothioate-modified antisense ODN against c-*MYC* have been reported[108,109]. Both studies showed safety of intracoronary application of the drug, but no objective clinical responses.

Oligonucleotides as immunological adjuvants

Over the past several years, it has become increasingly appreciated that several types of immune cell have pattern-recognition receptors that can distinguish prokaryotic DNA from vertebrate DNA[110]. This is apparently accomplished by the ability of these receptors to recognize unmethylated CpG dinucleotides in certain base contexts (CpG motifs)[111]. Bacterial DNA, or more germane to this discussion, synthetic oligodeoxynucleotides that contain these unmethylated CpG motifs, can activate immune responses that have evolved to protect the host against infections. Responses of this type are similar to T-helper type 1 (T_H1)-cell responses, and lead to activation of natural killer (NK) cells, dendritic cells, macrophages and B cells[112]. CpG DNA-induced immune activation has been shown to protect certain hosts against infection, either alone, or in combination with vaccines. It is reasonable to suppose, then, that CpG-containing oligonucleotides might prove to be effective adjuvants for the immunotherapy of cancer, and for boosting immune responses to antigens that are less efficient in this regard, but to which one would like to immunize a host[113].

The most recent application of this principle was reported in abstract form at the December 2001 meeting of the American Society of Hematology, where preliminary results from a clinical trial in which the

RESTENOSIS
A reduction in lumenal size after an inter-arterial coronary intervention.

Table 2 | **Current and planned clinical trials with antisense oligonucleotides and ribozymes**

Product	Diseases	Company
Anti-c-MYC (AS)	Cardiovascular restenosis, Phase II	AVI Biopharma
EPI 2010 (AS against adenosine A1 receptor)	Asthma, Phase II	EpiGenesis Pharmaceuticals
Genasense (AS against BCL2)	Haematological malignancies Solid tumours, Phase III	Genta
GTI 2040 (AS against ribonucleotide reductase)	Solid tumours, Phase I and II	Lorus Therapeutics
HGTV (AS against HIV)	HIV, Phase II	Enzo Biochem
CpG molecules	Solid tumors Infectious diseases, Phase I/II	Coley Pharmaceutical Group
Angiozyme (Ribozyme against VEGFR1)	Breast and colon cancer, Phase II	Ribozyme Pharmaceuticals
Heptazyme (Ribozyme against HCV)	HCV, Phase I	
Herzyme (Ribozyme against HER2)	Breast and ovarian cancer, Phase I	
ISIS 3521 (PKC-α) ISIS 5132 (c-RAF) ISIS 2503 (h-RAS) G 3139 (BCL2) GEM 231 (PKA)	NSCLC, NHL, Phase III Solid tumours, Phase II NSCLC, Phase II NHL, Phase II/III PKA, Phase II	ISIS Pharmaceuticals

AS, antisense; BCL2, B-cell lymphoma protein 2; CpG, unmethylated CpG dinucleotides; HCV, hepatitis C virus; HER2, tyrosine-kinase growth-factor receptor, also called c-ERBB2; HIV, human immunodeficiency virus; c-MYC, myelocytomatosis viral oncogene homologue; NHL, Non-Hodgkin's lymphoma; NSCLC, non-small-cell lung cancer; PKA, protein kinase A; PKC-α; protein kinase C-α; VEGFR1, vascular-endothelial-growth-factor receptor 1.

safety and efficacy of a CpG adjuvant was investigated in 16 patients with non-Hodgkin's lymphoma were reported[114]. Analysis of the data accrued at the time of submission indicated that the oligonucleotide increased the number and activity of NK cells in treated patients, and 2 out of 16 treated patients achieved partial remission. The study is continuing, and a follow-on trial of the CpG oligonucleotide in combination with rituximab is being planned.

Problems in need of solution

Nucleic-acid-mediated gene silencing has been used with great success in the laboratory[107,115–117], and this strategy has also generated some encouraging results in the clinic[90,93,96,118,119]. Nevertheless, it is widely appreciated that the ability of nucleic-acid molecules to modify gene expression *in vivo* is quite variable, and therefore wanting in terms of reliability[120,121]. Several issues have been implicated as a root cause of this problem, including molecule delivery to targeted cells and specific compartments within cells, and identification of sequence that is accessible to hybridization in the genomic DNA or RNA[2]. Intuitively, DNA accessibility is limited by compaction of nuclear material and transcription activity of the gene target. Formal approaches for solving this problem have not been widely discussed. In mRNA, sequence accessibility is dictated by internal base pairing and the proteins that associate with the RNA in a living cell. Attempts to accurately predict the *in vivo* structure of RNA have been fraught with difficulty[122]. Accordingly, mRNA targeting is largely a random process, which accounts for the many experiments in which the addition of an antisense nucleic acid yields no effect on expression. Several approaches to this problem have been tried, including trial-and-error 'walks' down the mRNA[123], computer-assisted modelling of RNA structure[124], hybridization of RNA to random oligonucleotides arrayed on glass slides[125,126] and variations on the theme of using random oligonucleotide libraries to identify RNase H cleavable sites, in the absence or presence of crude cellular extracts[127,128]. Recent work from this laboratory indicates that self-quenching reporter molecules might be useful for solving *in vivo* RNA structure[129], but the reliability and usefulness of this approach remain to be proven.

Another problem in this field is the limited ability to deliver nucleic acids into cells and have them reach their target[120]. Without this ability, it is clear that even an appropriately targeted sequence is not likely to be efficient. As a general rule, oligonucleotides are taken up primarily through a combination of adsorptive and fluid-phase endocytosis[130,131]. After internalization, confocal and electron microscopy studies have indicated that the bulk of the oligonucleotides enter the endosome–lysosome compartment, in which most of the material becomes either trapped or degraded. Biological inactivity is the predictable consequence of these events. Nevertheless, oligonucleotides can escape from the vesicles intact, enter the cytoplasm and then diffuse into the nucleus, where they presumably acquire their mRNA, or in the case of decoys, protein target[131,132–134]. Delivery technologies continue to improve, so it is likely that present methods, and/or other evolving technologies, will be used successfully to deliver optimized nucleic acids to their cellular targets[135,136]. Indeed, it is our hypothesis that development of

effectively targeted and efficiently delivered nucleic-acid molecules will lead to important advances in the diagnosis and treatment of human malignancies[93], and other diseases for which this class of molecule has been proposed to be effective.

In addition to delivering and targeting oligonucleotides to the mRNA, we believe that other considerations might improve the efficacy of this strategy. In this regard, we suggest that the abundance and half-life of the target mRNA should also be considered when selecting a gene target. The *c-MYB* mRNA that we have chosen to target, as well as its encoded protein, has an estimated half-life of ~30–50 minutes[137,138]. By contrast, BCL2, for example, has a half-life that has been estimated at ~14 hours[139], and RAF and RAS have half-lives that are estimated to be >24 hours[140,141]. Attempts to eliminate these proteins from cells using oligonucleotides might therefore prove more difficult. Whether these considerations will apply to extremely long lived or endogenously expressed antisense vectors, remains to be seen. As the efficiency of these molecules for perturbing gene expression improves, an important consideration in target selection will be the relative selection in the target versus non-targeted tissue. The ability to target genetic polymorphisms, or cells affected by loss of heterozygosity, might be an effective solution to this problem[142]. Finally, another approach for improving the effectiveness of nucleic-acid drugs as anticancer agents that is under intense investigation is to combine them with more traditional therapeutic modalities. Although this might well prove useful, we strongly believe that it remains important to continue to explore strategies that are designed to promote more reliable and efficient gene silencing with oligonucleotides alone. As discussed above, a prime motivating force for developing these drugs is the hope for non-toxic therapies. Adding back chemotherapy, although perhaps useful in the short term, is in the end counterproductive to this specific goal, unless it can be used at significantly reduced dosages. So far, this has not been the case.

Conclusions

The concept of inhibiting gene expression with antisense nucleic acids developed from studies that were initiated almost a quarter of a century ago[13,14]. Despite the fact that the mechanism by which these molecules modulate gene expression is not always certain[12,128,143], clinical development of antisense compounds has proceeded to the point at which several nucleic-acid drugs have entered Phase I/II, and in a few cases, Phase III trials. Others are about to begin, or are in the late planning stages (TABLE 2). The original motivation for developing these molecules remains strong. The recent development of leukaemia cells that are resistant to the small-molecule inhibitor Gleevec provides another incentive. Although a cell might be able to evolve mutated proteins that evade a small-molecule protein inhibitor, this cannot happen if the mRNA that encodes that protein is no longer made. Accordingly, although only one antisense drug has received FDA approval so far[144], all of the investigators who have laboured long and hard in this field hope that the time to celebrate significant achievements in the clinic will shortly be forthcoming.

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Suppression of Gene Expression by Targeted Disruption of Messenger RNA: Available Options and Current Strategies

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ABSTRACT

At least three different approaches may be used for gene targeting including: A) gene knockout by homologous recombination; B) employment of synthetic oligonucleotides capable of hybridizing with DNA or RNA, and C) use of polyamides and other natural DNA-bonding molecules called lexitropsins.

Targeting mRNA is attractive because mRNA is more accessible than the corresponding gene. Three basic strategies have emerged for this purpose, the most familiar being to introduce antisense nucleic acids into a cell in the hopes that they will form Watson-Crick base pairs with the targeted gene's mRNA. Duplexed mRNA cannot be translated, and almost certainly initiates processes which lead to its destruction. The antisense nucleic acid can take the form of RNA expressed from a vector which has been transfected into the cell, or take the form of a DNA or RNA oligonucleotide which can be introduced into cells through a variety of means. DNA and RNA oligonucleotides can be modified for stability as well as engineered to contain inherent cleaving activity.

It has also been hypothesized that because RNA and DNA are very similar chemical compounds, DNA molecules with enzymatic activity could also be developed. This assumption proved correct and led to the development of a "general-purpose" RNA-cleaving DNA enzyme. The attraction of DNAzymes over ribozymes is that they are very inexpensive to make and that because they are composed of DNA and not RNA, they are inherently more stable than ribozymes.

Although mRNA targeting is impeccable in theory, many additional considerations must be taken into account in applying these strategies in living cells including mRNA site selection, drug delivery and intracellular localization of the antisense agent. Nevertheless, the ongoing revolution in cell and molecular biology, combined with advances in the emerging disciplines of genomics and informatics, has made the concept of nontoxic, cancer-specific therapies more viable then ever and continues to drive interest in this field. Stem Cells *2000;18:307-319*

INTRODUCTION

The notion that gene expression could be modified through use of exogenous nucleic acids derives from studies by *Paterson et al.* who first used single-stranded DNA to inhibit translation of a complementary RNA in a cell-free system in 1977 [1]. One year later, *Zamecnik* and *Stephenson* noted that a short (13nt) DNA oligonucleotide reverse complementary in sequence (antisense) to the Rous sarcoma virus could inhibit viral replication in culture [2]. This observation is credited as being among the first to suggest the therapeutic utility of antisense nucleic acids, a concept which ultimately led to the awarding of a Lasker Prize in Medicine to *Dr. Zamecnik*. In the mid 1980s, the existence of naturally occurring antisense RNAs and their role in regulating gene expression was demonstrated [3-5]. These observations were particularly important because the

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fact that naturally occurring antisense nucleic acids played a role in regulating gene expression lent support to the belief that exogenously introduced reverse complementary nucleic acids might be utilized to manipulate gene expression in living cells. These seminal papers, and the literally thousands which have followed, have stimulated the development of technologies employing nucleic acids to manipulate gene expression. Virtually all available methods rely on some type of nucleotide sequence recognition for targeting specificity, but differ where and how they perturb the flow of genetic information [6]. Simply stated, strategies for modulating gene expression may be thought of as being targeted to the gene itself, or to the gene's messenger RNA (mRNA). Since this review will be focused on strategies aimed at disrupting the use of mRNA, antigene strategies will be addressed only briefly and mainly for the sake of completeness.

Antigene Strategies

At least three different approaches may be utilized for direct gene targeting. The "gold standard" is the gene "knock-out" achieved by homologous recombination [7, 8]. This approach results in the actual physical disruption of the targeted gene as a result of crossover events which occur during cell division between the targeting vector and the gene selected for destruction (Fig. 1A). Homologous recombination is extremely powerful, but the technique is hampered by the fact that it remains inherently inefficient, time-consuming, and expensive. While improvement in the efficiency of this process has been achieved [9, 10], this is a method which remains restricted to use in cell lines and animal models, if for no other reason than selection is required to find the cells in which the desired events have taken place. In clinical situations where high efficiency gene disruptions are required, it seems unlikely that this approach will serve as a useful therapeutic modality anytime in the foreseeable future.

A second option for gene targeting employs synthetic oligodeoxynucleotides (ODN) capable of hybridizing with double-stranded DNA [11-13]. Such hybrids are typically formed within the major groove of the helix, though hybridization within the minor groove has also been reported [14]. In either case, a triple-stranded molecule is produced, hence the origin of the term triple helix-forming oligodeoxynucleotide (TFO) (Fig. 1B). TFOs do not destroy a gene but prevent its transcription either by preventing unwinding of the duplex or preventing binding of transcription factors to the gene's promoter. TFO sequence requirements are based on the need for each base comprising the TFO to form two hydrogen bonds (Hoogsteen bonds) with its complementary base in the duplex. This constrains TFOs to hybridization with the purine bases composing polypurine-polypyrimidine tracks within the DNA. The targeting efficiency of TFOs is further constrained by a number of factors, including need for divalent cations, and perhaps most importantly, by access to DNA compacted within the chromosome structure. Recent experiments from *Wang et al.* and *Kochetkova et al.* have provided evidence that triple helix formation can occur in living cells, suggesting that these difficulties may ultimately be overcome [15-17]. If shown practical, it has also been postulated that TFOs may prove useful in the treatment of certain genetic disorders such as sickle cell anemia, and hemophilia B, where their ability to trigger repair mechanisms might be used to correct single base pair mutations responsible for the disease [15, 18-20].

Final approaches worth mentioning are the use of specific nucleic acid sequences to act as "decoys" for transcription factors [21, 22], and the use of polyamides and

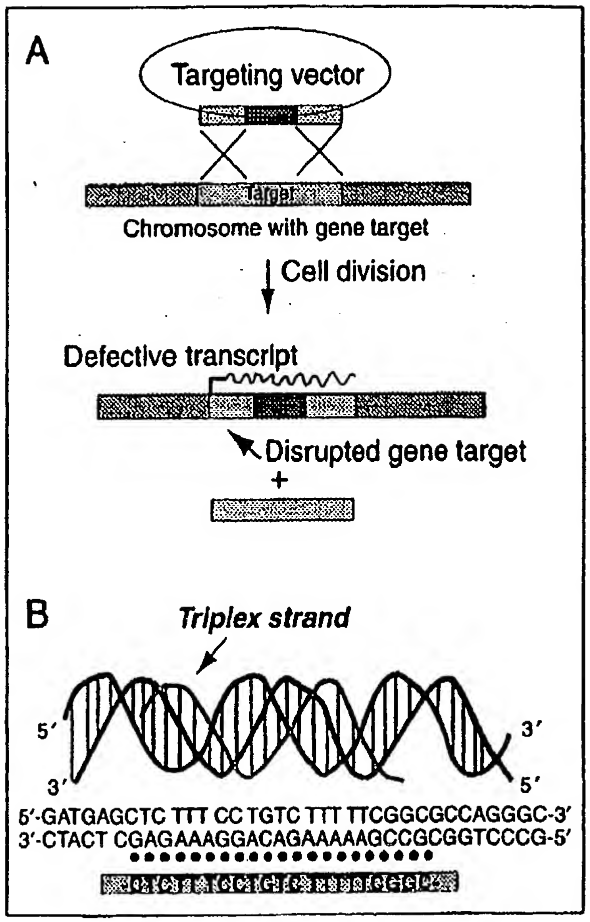

Figure 1. A) Targeting vector; B) Triplex strand. Adapted from [6].

other natural DNA-binding molecules called lexitropsins, that bind to specific bases in the minor groove of DNA [23, 24]. The use of decoy molecules evolves from the knowledge that transcription factor proteins recognize and bind specific DNA sequences. In theory then, it is possible to synthesize nucleic acids which will effectively compete with the native DNA sequences for available transcription factor proteins in vivo. If effective, the rate of transcription of the genes dependent on the particular factor involved will diminish. Unless single gene transcription factors can be identified, it is difficult to conceive how this approach, though potentially effective for controlling cell growth, can be made gene-specific. The polyamide approach may prove feasible since sequence-specific molecules can likely be designed and it appears that molecules of this type can easily access DNA within the chromosomes [23-25].

Anti-mRNA Strategies

A gene may be effectively "silenced" by destabilizing its mRNA, thereby preventing synthesis of the protein it encodes. Targeting mRNA, while less favorable than antigene strategies from a stoichiometric point of view, is nonetheless attractive because mRNA is in theory more accessible. Three basic strategies have emerged for this purpose. One employs an oligonucleotide that acts as an alternate binding site, or "decoy," for protein-stabilizing elements that normally interact with a given mRNA [26, 27]. By attracting away mRNA-stabilizing protein, the decoy induces instability, and ultimately destruction, of the mRNA. A newly developing approach is to affect RNA interference (RNAi) or post-transcriptional gene silencing [28, 29]. RNAi employs a gene-specific double-stranded RNA which, when introduced into a cell, leads to diminution of the targeted mRNA. The actual mechanism whereby this is accomplished is presently unknown but is under intense investigation with several clues being deciphered already [30, 31] including size and necessity for processing of the targeting dsRNA. In *C. elegans* and Drosophila this is a highly reproducible method for disrupting gene expression. Some reports suggest that this technique can be adapted for use in mammalian cells [32], but this remains uncertain at the moment. Finally, there is the more familiar, and more widely applied "antisense" strategy. We will focus on the latter.

Antisense (reverse complementary) nucleic acids are introduced into a cell in hopes that they will form Watson-Crick base pairs with the targeted gene's mRNA. As stated above, duplexed mRNA cannot be translated, and almost certainly initiates processes which lead to its destruction. The antisense nucleic acid can take the form of RNA expressed from a vector which has been transfected into the cell [33], or take the form of a DNA or RNA oligonucleotide which can be introduced into cells through a variety of means. DNA and RNA oligonucleotides can be modified for stability as well as engineered to contain inherent cleaving activity [34, 35]. A number of these issues will be discussed in more detail in the sections below.

Antisense Oligonucleotides (AS-ONs)

AS-ONs are short stretches of nucleotides that are complementary to a region of targeted mRNA and can specifically suppress expression of that particular transcript. The following discussion will focus on the fundamental concepts concerning AS-ONs and their mechanisms of action. Examples of AS-ON use in experimental and clinical settings have been recently reviewed [36-38].

The exact mechanism of AS-ON action remains unclear, but it is known to be different for various types of AS-ONs. Generally, these molecules block gene expression by hybridizing to the target mRNA, resulting in subsequent double-helix formation. This process can occur at any point between the conclusion of transcription and initiation of translation, or even possibly during translation. Disruption of splicing, transport, or translation of the transcripts are all possible mechanisms, as is stability of transcript. Therefore, a major question is whether AS-ONs are most effective in the cytoplasm or nucleus. In the case of antisense oligodeoxyribonucleotides (AS-ODNs), cellular RNase H is able to bind to the DNA-RNA duplex and hydrolyze the RNA, resulting in increased transcript turnover. Any modification to the deoxy moiety at the 2′-sugar position prohibits RNase H action.

Modified AS-ONs or AS-ON analogs are often employed for in vivo antisense applications due to their increased stability and nuclease resistance. A longer serum half-life ensures that the AS-ON has ample time to reach and interact with its target mRNA. Phosphorothioate AS-ODNs are most widely used due to their long serum half-life and the fact that they are a suitable RNase H substrate. However, phosphorothioates display high affinity for various cellular proteins, which can result in sequence-nonspecific effects [39, 40]. Furthermore, high concentrations of phosphorothioates inhibit DNA polymerases and RNase H, which may render them ineffective as antisense agents [41]. Interestingly, many AS-ONs with 2′-modifications with groups such as O-methyl, fluoro, O-propyl, O-allyl, or many others exhibit greater duplex stability with their target mRNA along with antisense effects independent of RNase H (Fig. 1). These modifications create bulk at the 2′ position, causing steric hindrance to play a significant role in increasing nuclease resistance. Nucleotide analogs

generally are also nuclease-resistant and often demonstrate superior hybridization properties due to modified backbone charge, although they usually are not acceptable substrates for RNase H. One example is peptide nucleic acid (PNA) where the sugar-phosphate moiety has been replaced by 2-aminoethyl glycine carbonyl units [42]. To these units are attached nucleotide bases spaced equally apart to DNA nucleotide bases. Instead of phosphodiester linkages between nucleotides, peptide bonds join the monomers to create a backbone neutral in charge. Not only do PNA oligomers hybridize to complementary DNA and RNA by Watson-Crick base pairing, they do so more quickly [43] and with greater affinity [42-44] because of the neutral backbone. In addition, PNAs are better at discriminating between base pair mismatches [44] and form less nonsequence-specific associations with proteins than phosphorothioate oligonucleotides [45]. Positive charges can also be introduced to backbone structure as in the case of (2-aminoethyl)phosphonates. Increased stability of duplex formation with both RNA and DNA has been reported with hybrid stability being more pH-dependent and less salt-dependent than natural RNA or DNA duplexes [46].

Some insight into the mechanism of AS-ON action has emerged recently through the work of *Baker* and colleagues (unpublished). Differences in ability to inhibit gene expression occur when either 2′-modified AS-ONs or 2′-unmodified AS-ONs are targeted to the exon 9 region of interleukin 5 (IL-5). Two forms of IL-5 exist: a soluble IL-5 lacking the exon 9 region, and a membrane-bound form, which contains exon 9. When unmodified AS-ONs are targeted to exon 9 of the IL-5 transcript, the expression of both membrane-bound and soluble IL-5 is inhibited. However, 2′-modified AS-ONs only suppress membrane-bound IL-5 expression. These observations seem to suggest that RNase H-dependent antisense effects are a nuclear event prior to splicing, whereas RNase H-independent oligonucleotides may affect splicing in transcript processing or may suppress gene expression after splicing has taken place. Additional evidence demonstrates that in the absence of RNase H activity, antisense effects may be a result of interference with translational initiation complex formation for certain types of 2′-modified AS-ON such as 2′-O-(2-methoxy) ethyl AS-ONs [47].

Ribozymes

Naturally occurring ribozymes are catalytic RNA molecules that have the ability to cleave phosphodiester linkages without the aid of protein-based enzymes. This property has been exploited to specifically inhibit gene expression by targeting mRNA for catalytic cleavage especially in viral, cancer, and genetic disease therapeutics [48]. Similar to AS-ONs, ribozymes bind to substrate RNA through Watson-Crick base pairing, which offers sequence-specific cleavage of transcripts. Ideally, these agents should trigger enhanced transcript turnover as compared to RNase H-mediated AS-ON degradation of transcripts, considering ribozymes act through bimolecular kinetics (association of ribozyme and target transcript) whereas RNase H-dependent AS-ONs rely on trimolecular kinetics (association of AS-ON, target transcript, and RNase H). Since ribozymes are RNase H-independent, 2′-modifications to increase stability do not diminish antisense effects and experiments have shown some modifications do not attenuate catalytic ability [49]. Unlike AS-ONs, ribozymes can be expressed from a vector, which offers the advantage of continued production of these molecules intracellularly [50, 51]. However, stable transformation of cells in vivo has its own complications and will not be discussed in this review.

If ribozymes are to perform effectively as "enzymes," they must not only bind substrate RNA but also dissociate from the cleavage product in order to act on additional substrates. Studies suggest that in some cases, dissociation of cleavage product may be the rate-limiting step [52, 53]. Furthermore, some ribozymes require high divalent metal ion concentrations for efficient substrate cleavage, which may limit their use in intracellular environments [54]. All of these concerns need to be addressed and overcome in order for ribozymes to have a future in medical therapy. Two ribozymes, the hammerhead ribozyme and the hairpin ribozyme, have been extensively studied due to their small size and rapid kinetics. Their application has been recently reviewed in several publications [55-59].

Hammerhead Ribozymes

The hammerhead ribozyme consists of a highly conserved catalytic core, which will cleave substrate RNA at NUH triplets 3′ to the H, where N is any nucleotide, U is uracile, and H is any nucleotide but guanidine (Fig. 2) [34]. In fact RNA cleavage may be less restricted since recent studies demonstrate exceptions to the "NUH" rule. Investigators have established that cleavage can actually occur 3′ to any NHH triplet [59]. Furthermore, in vitro selection protocols have made it possible to screen for ribozymes with various cleavage specificities including one that cleaves at AUG sites [60]. Thus, the limitations for sequence specificity of triplet-cleavage sites on the target RNA are less than previously thought. In addition to the catalytic core, a particular cleavage site in a target RNA can be specifically recognized by the hammerhead ribozyme arms. By creating complementary sequences in the arms to sequences flanking the cleavage site, the ribozyme will hybridize specifically to the RNA of interest.

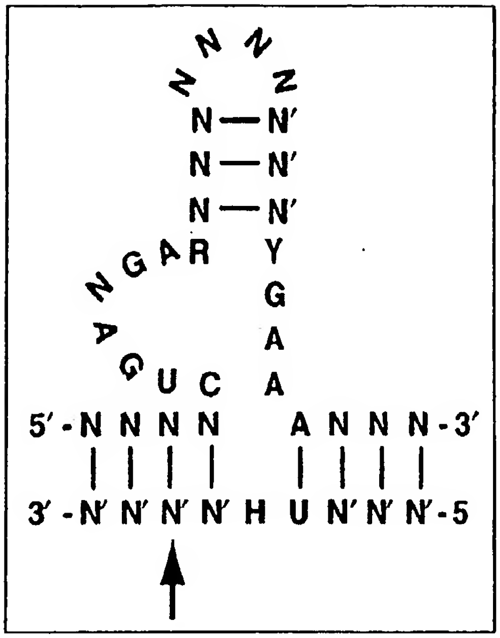

Figure 2. Hammerhead ribozyme (top strand) hybridized to target RNA. Arrow indicates position of cleavage. N = A, G, T, or C; N' = nucleotide complementary to N; H = any nucleotide but G; Y = pyrimidine nucleotide; R = purine nucleotide complementary to Y. Adapted from [56].

Subsequent cleavage will then be directed towards that particular position.

The catalytic ability of hammerhead ribozymes is dependent on the presence of divalent metal ions, of which magnesium is most often used in vitro. It is postulated that the ions not only participate in RNA folding but also in the cleavage step itself [54]. As mentioned previously, studies indicate that catalytic activity requires relatively high Mg^{2+} concentrations compared to the intracellular environment. This characteristic could be problematic in applying the hammerhead ribozyme to an in vivo setting where intracellular Mg^{2+} concentrations are 5- to 10-fold lower than optimal in vitro conditions.

Much evidence supports diminished mRNA levels and gene products directly due to hammerhead ribozyme delivery. There is also indication from reverse transcriptase-polymerase chain reaction (RT-PCR) and reverse ligation (RL)-PCR protocols of messenger RNA cleavage at the targeted position in cellular RNA extracts [61, 62]. Recently, more potent ribozyme-mediated effect on viral and cancer cell growth compared to noncatalytic RNAs was reported [63, 64]. However, in some instances, hammerhead ribozymes have not proven to be more effective than AS-ON and instead give equal degrees of gene suppression. Likewise, inactive control ribozymes where antisense binding can occur, but catalytic ability has been abolished, give similar levels of gene inhibition when compared to fully catalytic hammerhead ribozymes, suggesting that the catalytic core, in some instances, plays little role in enhancing antisense effects [65]. Only further detailed studies will reveal the true utility of hammerhead ribozymes.

Hairpin Ribozymes

The natural hairpin ribozyme is derived from a negative strand of the tobacco ringspot virus satellite RNA. Work on engineered hairpin ribozymes has resulted in a broader range of cleavage-sequence specificity. In general, a phosphodiester cleavage takes place 5' to the G in the sequence NGUC where N is any nucleotide [66], although recent studies have shown even less restriction on sequence requirements for cleavage [67].

The overall structure of the hairpin ribozyme consists of two domains connected by a hinge section (Fig. 3). One domain binds the substrate RNA to form two helical regions separated by a pair of single-stranded loops. Cleavage occurs within the single-stranded area of the substrate RNA. The other domain is similar in structure except the helixes are formed from the ribozyme folding back onto itself. The most important sequences for cleavage activity are those within the single-stranded regions where almost every nucleotide is conserved, while the helical portions can be almost any sequence as long as there is double-helix formation [58]. The hinge allows the two domains to be flexible relative to one another in space so that the two can dock together in an antiparallel orientation required for cleavage catalysis [68, 69].

Both the hairpin and hammerhead ribozymes require metal ions for cleavage catalysis. In the hammerhead ribozyme, metal ions are believed to be involved directly in the cleavage step [54, 70], whereas metal ions have not been implicated to be directly involved with cleavage for the hairpin ribozyme [71]. The metal ions in hairpin ribozymes may instead play an important role in ribozyme structure [72]. Fluorescence resonance energy transfer (FRET) studies on docking of the two domains show that docking is metal-dependent, but almost any metal will suffice even though they may not support cleavage [73]. In addition, docking is not the rate-limiting step, and since metal ions are not thought to be involved in the chemical cleavage step, it can only be assumed that there is a slower step in between docking and chemical cleavage.

One of the advantages offered by hairpin ribozymes is their unique ion-dependence for catalytic action. One group has shown that aminoglycoside antibiotics with at least four amino groups are able to both support and to

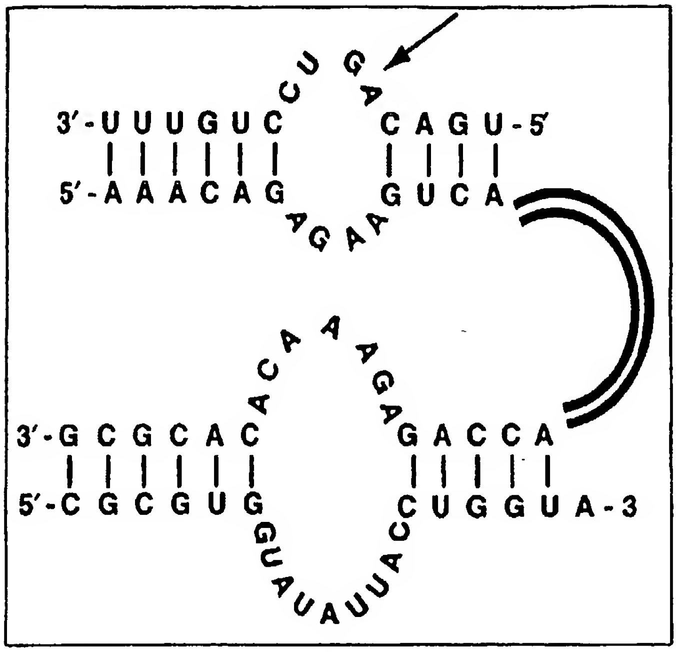

Figure 3. Hairpin ribozyme in the docked position. The two loop regions associate with each other in order to cleave the substrate RNA. Arrow indicates position of cleavage. Adapted from [58].

inhibit hairpin ribozyme cleavage depending on metal ion conditions [74]. In the presence of magnesium, aminoglycoside antibiotics inhibit ribozyme cleavage with the degree of inhibition depending on the binding affinity of the antibiotic to the ribozyme. However, in the absence of metal ions, aminoglycoside antibiotics prove to assist cleavage with an optimum reaction condition at pH 5.5 and poorer kinetics as the pH is increased, exactly opposite to trends observed for magnesium. In this case, the metal ions are most likely being replaced by the amino groups of these antibiotics.

Polyamines such as spermidine and spermine have also been reported to support hairpin ribozyme cleavage ability. In the absence of magnesium, spermidine allows the cleavage reaction to persist at very slow kinetics compared to magnesium alone [72]. However, spermine alone gives very efficient cleavage of RNA comparable to that of magnesium, and when in the presence of low magnesium concentrations similar to intracellular conditions, spermine displays considerable increase in cleavage rates [74]. The fact that spermine is the major polyamine in eukaryotic cells may explain why the hairpin ribozyme has shown remarkable intracellular cleavage activity in mammalian cells and may make future therapeutic endeavors with the hairpin ribozyme much easier [75].

DNAzymes

While investigating ways to improve the function of ribozymes, *Breaker* and *Joyce* made the assumption that because RNA and DNA are very similar chemical compounds, DNA molecules with enzymatic activity could also be developed [76]. This assumption proved correct and led to the development of a "general-purpose" RNA-cleaving DNA enzyme [77]. The molecule was identified from a library of >1,000 different DNA molecules by successive rounds of in vitro selective amplification based on the ability of individual molecules to promote Mg^{2+}-dependent, multiturnover, cleavage of an RNA target.

The selected molecule was named the "10-23 DNA enzyme," because it was derived from the 23rd clone obtained after the 10th round of selective amplification [77]. The "10-23 DNA enzyme" is composed of a catalytic domain of 15 deoxynucleotides, flanked by 2 substrate-recognition domains of ~8 nucleotides each (Fig. 4). The recognition domains provide the sequence information required for specific binding to an RNA substrate. They also supply the binding energy required to hold the RNA substrate within the active site of the enzyme. It is straightforward that by appropriately designing the flanking sequences, the DNAzyme can be made to cleave virtually any RNA that contains a purine-pyrimidine junction.

The attraction of DNAzymes over ribozymes is that they are very inexpensive to make and that because they are composed of DNA and not RNA, they are inherently more stable

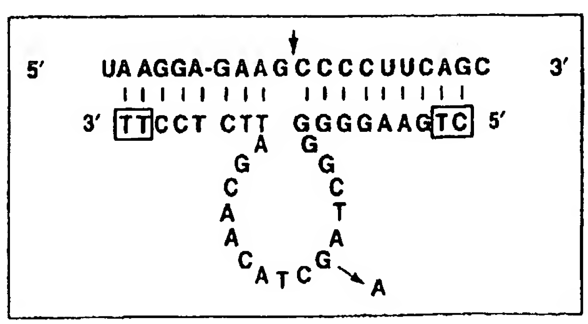

Figure 4. Complex formed by an mRNA (top strand) and a "10-23" DNAzyme (bottom strand). Vertical arrow indicates the mRNA cleavage site. Replacement of G by A within the catalytic core of the DNAzyme (diagonal arrow) will eliminate its catalytic activity. Adapted from [77].

than ribozymes. Nevertheless, DNAzymes must ultimately overcome the same problems faced by ribozymes and oligonucleotides if they are to be effective in cellular systems (see below). These are stability, ability to be targeted to the cell of interest, ability to achieve sufficient intracellular concentration to cleave to the targeted mRNA, ability to hybridize with their mRNA target, and lack of toxicity. In this regard, many of the chemical modifications employed to stabilize ODNs can be incorporated into the 10-23 DNA enzyme without loss of activity. There is a suggestion from recent reports that issues of intracellular concentration and target hybridization may also be solvable [78, 79].

Application of the "Antisense" Strategy

Although antisense interference methods appear impeccable in theory, many additional considerations must be taken into account in applying the strategy in living cells. Since both AS-ONs and ribozymes are considered oligonucleotides, quite often similar solutions can be offered to address the problems encountered. As mentioned earlier, increasing stability of antisense agents can be easily achieved through nucleotide modifications or analogs. However, additional considerations crucial to reliable experimental outcome include mRNA site selection, drug delivery, and intracellular localization of the antisense agent.

mRNA Site Selection

Within living cells, transcripts exist in low energy conformations in which secondary structures dominate in folding the linear polymer. In addition, interactions with cytoplasmic proteins produce further structural properties. The end result is that much of the mRNA sequence is hidden and only partial sequences within the total mRNA length are accessible for hybridization. RNA folding programs that generate three-dimentional folding patterns based on free energy calculations often give an unreliable depiction for in vivo relevance. Therefore, a good empirical method to probe for suitable sites is necessary.

A system to probe for suitable sites in mRNA for AS-ON or ribozyme-targeting has recently been established using RNase H cleavage as an indicator for accessibility of sequences within transcripts [80]. A mixture of ODNs that are complementary to certain regions of a transcript is added to cell extracts and exposed to RNase H. RT-PCR of the transcript can then be used to show which ODNs actually had access to the transcript and hybridized in order to create an RNase H-vulnerable site. Combining this methodology with computer-assisted sequence selection may enhance this approach as well [81].

Another technique currently being tested is the use of molecular beacons for site selection (*Gewirtz et al.*, unpublished). These molecules are ODNs with the ability to form stem-loops where the loops are targeted to regions of the transcript [82]. The stems have a fluorophore linked to either the 5′ or 3′ end and a quencher molecule is attached to the other so that in the stem-loop configuration, fluorescence is not observed due to the proximity of the quencher molecule to the fluorophore. However, when hybridization proceeds, the act of forming a double helix between the loop and the transcript causes unfolding of the stem-loop and brings the quencher and fluorophore apart in space. Thus, fluorescence should increase as a result of hybridization. Currently, these molecules are being applied to probe for accessible sites within mRNA with very encouraging results (*Jen* and *Gewirtz*, unpublished).

Delivery

One of the major limitations for the therapeutic use of AS-ONs and ribozymes is the problem of delivery. Import of these compounds into cells can be accomplished by exogenous delivery in which presynthesized oligonucleotides come in direct contact with the plasma membrane, resulting in subsequent cellular uptake [83]. Naked oligonucleotides are poorly incorporated into cells in this fashion and often require a vehicle for efficient delivery. In tissue culture, many classes of compounds have been used as delivery vehicles including cationic liposomes, cationic porphyrins, fusogenic peptides, and artificial virosomes. These compounds share the characteristic of forming complexes with oligonucleotides through electrostatic interactions between the negatively charged oligonucleotide phosphate groups and positive charges contained by the vehicles themselves. In addition, some degree of protection from nuclease degradation is conferred to the oligonucleotide when associated with such delivery vehicles. Other strategies including cell permeabilization with streptolysin-O and electroporation have been used [84] but are restricted in utility for clinical settings. Presently, some success has been achieved in tissue culture, but efficient delivery for in vivo animal studies remains questionable.

Cationic lipids form stable complexes with oligonucleotides, which exhibit improved cellular uptake [85-87]. The result is enhanced antisense activity. Further studies indicate that phosphorothioated ODNs dissociate from cationic lipids before entering the nucleus where it is free to hinder target transcript function [88]. These compounds have proven to be quite effective in cell culture and have been commercialized, but their relatively high cytotoxic properties may restrict their use.

Alternatives to cationic lipids are being explored. Recently, cationic porphyrins have proven to be effective vehicles for AS-ONs in tissue culture [89, 90]. Two cationic porphyrins used by *Benimetskaya* and colleagues, tetra(4-methylpyridyl) porphyrin (TMP) and tetraanilinium porphyrin (TAP), demonstrate properties important for AS-ON delivery. 5′-fluorescein-labeled phosphorothioates show that both TMP and TAP more efficiently deliver AS-ONs into cells than naked AS-ONs. Nuclear fluorescence is observed after porphyrin/AS-ON complex exposure to cells while fluorescein labeled AS-ONs alone are taken up into vesicular structures. Thus, cationic porphyrins not only help AS-ON delivery into the cell, but they are also able to localize the AS-ON in the nucleus where mRNA and RNase H are present. FRET studies on the ability of cationic porphyrins to quench 5′-fluorescein-labeled phosphorothioates suggest intracellular dissociation of the oligonucleotide from the porphyrin.

Fusogenic peptides form peptide cages around oligonucleotides in order to boost oligonucleotide uptake. Many of these peptides contain polylysine residues, which cause membrane destabilization [91]. Others are derived from viral proteins such as the fusion sequence of HIV gp41 [92] and hemagglutinin envelop protein [93, 94]. Generally, these agents are less cytotoxic than lipids but are still able to achieve similar delivery efficacy. Artificial virosomes are another class of delivery vehicles which take advantage of the natural ability of a virus to gain entry into cells. Reconstituted influenza virus envelopes known as virosomes can fuse with endosomal membranes after internalization through receptor-mediated endocytosis [95]. Recently, cationic lipids have been incorporated into virosome membranes to further aid delivery [96, 97].

Finally, *Dheur* and colleagues have noted that while oligonucleotides delivered with lipofectins usually do not elicit antisense activity (likely because cationic lipid formulations do not protect unmodified oligonucleotides from nuclease degradation), a cationic polymer, polyethylenimine (PEI) [98], improves the uptake and antisense activity of antisense phosphodiester oligodeoxynucleotides (PO-ODN) [99]. Interestingly, PEI-phosphorothioate (PS) ODN particles were efficiently taken up by cells but PS-ODN did not dissociate from the carrier. These investigators suggested that the low cost of PEI compared with cytofectins, the increased affinity for target mRNA and decreased affinity for proteins of PO-ODN compared with PS-ODN might make the use of PEI-PO-ODN very attractive.

Localization

In order for AS-ONs or ribozymes to suppress gene expression, they must be colocalized to the same intracellular compartment as their target mRNA. Intracellular trafficking seems to play an important role in the fate of these molecules since their spatial distribution does not correspond to simple diffusion. Many factors determine localization patterns of AS-ON and ribozymes including the antisense agent itself, delivery vehicle, and targeted cell type. In addition, evidence for cell cycle-dependent localization patterns has been reported with nuclear localization predominantly in the G_2/M phase [100].

mRNAs can exist in several cellular compartments including the cytoplasm, nucleus, and nucleolus. It remains unclear as to where oligonucleotides should be directed for most efficient antisense activity to occur, although endosomal localization usually predicts ineffective antisense response. The optimal site for mRNA degradation may be dependent on the type of antisense agent used [47]. Recently, ribozymes attached to small nucleolar RNAs (snoRNAs) called snoribozymes exhibited nearly 100% efficiency in cleaving a target RNA also localized to the nucleolus by snoRNA attachment [101]. Even though this particular experiment is based on cleavage of an artificial substrate, the expanding roles associated with the nucleolus may prove the nucleolus to be an important site to target mRNA degradation [102]. In another study, antisense RNA inserted within a variable region of ribosomal RNA (rRNA) proved to heighten ribozyme efficiency and may be due to colocalization of rRNA with mRNA [103].

Antisense Drug Design

Certain issues to be aware of concerning antisense experimental design are quite important to the consistent and efficacious outcome of inhibiting gene expression. Even when the above considerations regarding the potential problems of antisense experiments are addressed, other factors may come into play especially involving antisense drug design. Only two will be mentioned here: formation of G quartets and chirality of modified oligonucleotides. Purine-rich oligonucleotides, especially ones containing four consecutive guinine residues, have a tendency to form stable tetrameric structures under physiologic conditions [104]. The guinosines of single-stranded oligonucleotides are not restrained in space by rigid double-helix structure and can therefore form various hydrogen bonds not observed in Watson-Crick base pairing. Tetraplexes known as G quartets arise as a result. Dissociation rates of these structures may be quite slow and may prevent hybridization of AS-ONs or ribozymes to their target transcript, rendering them ineffective as antisense agents. However, the absence of G quartet structures at 37°C under cellular salt conditions could mean that G quartet formation is irrelevant at physiologic temperatures [105].

Interestingly, nonsequence-specific gene inhibition by phosphorothioate oligonucleotides containing tetraguinosine tracts prove aptameric properties can play an important role in gene inhibition for some sequences of ONs [106].

Another important aspect to consider is the issue of chirality for certain oligonucleotides. Unmodified phosphodiester oligonucleotides do not have a chiral center at the phosphorous position. However, when a terminal oxygen of the phosphate is replaced by a sulfur, as in PS-ONs, the phosphorous gains chirality. The digestion kinetics of PS-ONs by 3′-exonucleases display bi-exponential decay with a fast and slow phase of digestion. These phases are due to stereoselectivity of the 3′-exonucleases on the chiral phosphorothioate center [107]. A 25-mer containing a 3′-terminal internucleotide linkage in the S-configuration degrades 300-fold slower than the same 25-mer with an R-configuration phosphorothioate linkage.

Conclusions

The ongoing revolution in cell and molecular biology, combined with advances in the emerging disciplines of genomics and informatics, has made the concept of non-toxic, cancer-specific therapies more viable then ever. The recent development of a relatively specific biochemical inhibitor of the bcr/abl protein tyrosine kinase in patients with chronic myelogenous leukemia is a stunning example of this principle [108]. For therapies focused on direct replacement, repair, or disabling of disease-causing genes, progress has been much slower and a successful equivalent to the biochemical bcr/abl inhibitor has yet to be achieved. In the case of anti-mRNA strategies, it is hoped that the above discussion will have made the reasons for this clearer. Given the state of the art, it is perhaps not surprising that effective and efficient clinical translation of the antisense strategy has proven elusive. While a number of phase I/II trials employing ONs have been reported [109-116], virtually all have been characterized by a lack of toxicity but only modest clinical effects. A recent paper by *Waters et al.* describing the use of a bcl-2-targeted ON in patients with non-Hodgkin's lymphoma is typical in this regard [117, 118].

The key challenges to this field have been outlined above. It is clear that they will have to be solved if this approach to specific antitumor therapy is to become a useful treatment approach. A large number of diverse and talented groups are working on this problem, and we can all hope that their efforts will help lead to establishment of this promising form of therapy.

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Progress in Antisense Technology

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Key Words RNA, therapeutics, RNase H, RNAi, double-strand RNase

■ **Abstract** Antisense technology exploits oligonucleotide analogs to bind to target RNAs via Watson-Crick hybridization. Once bound, the antisense agent either disables or induces the degradation of the target RNA. Antisense agents can also alter splicing. During the past decade, much has been learned about the basic mechanisms of antisense, the medicinal chemistry, and the pharmacologic, pharmacokinetic, and toxicologic properties of antisense molecules. Antisense technology has proven valuable in gene functionalization and target validation. With one drug marketed, Vitravene®, and approximately 20 antisense drugs in clinical development, it appears that antisense drugs may prove important in the treatment of a wide range of diseases.

INTRODUCTION

Antisense technology exploits oligonucleotide analogs (typically 15–20 nucleotides) to bind to cognate RNA sequences through Watson-Crick hybridization, resulting in the destruction or disablement of the target RNA. Thus, antisense technology represents a new pharmacology. The "receptor," mRNA, has never before been considered in the context of drug receptor interactions. Prior to the advent of antisense technology, no medicinal chemistry had been practiced on oligonucleotides, the putative drugs. The basis of the drug receptor interaction, Watson-Crick hybridization, had never been considered as a potential binding event for drugs and put into a pharmacologic context. Finally, postbinding events such as recruitment of nucleases to degrade the receptor RNA had never been considered from a pharmacologic perspective.

A key to understanding antisense technology is to consider it in pharmacologic context. It is essential to understand the structure, function, and metabolism of the receptors for antisense oligonucleotides, and to consider their effects in the context of dose-response curves. In the future, advances in antisense biology and medicinal chemistry will improve pharmacologic behaviors.

EVALUATION OF ANTISENSE DRUGS

Elucidating the mechanism(s) of action of any drug is challenging, but drugs of novel structure and action such as antisense oligonucleotides present a particularly difficult challenge. For RNase H and double-strand RNase-activating oligonucleotides, demonstration of reduction of target mRNA abundance by Northern blot, RT-PCR, or RNase protection assays, or transcriptional assay analyses are required. Ideally, demonstration that the levels of closely related mRNAs are unaffected should be included. In brief, the following data-gathering steps should be taken with any new antisense oligonucleotide:

1. Generate dose-response curves in vitro using several cell lines and methods of in vitro delivery.
2. Generate dose-response curves in vivo and correlate the rank-order potencies in vitro and in vivo.
3. Perform "gene walks" for all RNA species and oligonucleotide chemical classes, i.e, evaluate the effects of ASOs designed to bind to 40–80 sites in a target RNA, then select the best.
4. Perform time courses.
5. Directly demonstrate the proposed mechanism of action by measuring the target RNA and/or protein.
6. Evaluate specificity and therapeutic indices via studies on closely related mRNA isotypes and appropriate toxicologic studies.
7. Use RNase H protection assays and transcriptional arrays to provide broader analyses of specificity.
8. Perform sufficient pharmacokinetics to define rational dosing schedules for pharmacologic studies.
9. When control oligonucleotides display surprising activities, determine the mechanisms involved.

MOLECULAR MECHANISMS OF ANTISENSE DRUGS

Occupancy-Only Mediated Mechanisms

Classic competitive antagonists are thought to alter biological activities because they bind to receptors, thereby preventing agonists from binding to them. Binding of oligonucleotides to specific sequences may inhibit the interaction of the RNA with proteins, other nucleic acids, or other factors required for essential steps in the intermediary metabolism of the RNA or its utilization by the cell. This can inhibit processes such as translation or splicing, or alter the metabolism of the RNA (for review see 1).

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Occupancy-Inducted Destabilization

Any oligonucleotide analog that can form a duplex with the target RNA that is a substrate for a nuclease may destabilize a target RNA. Two groups of enzymes have been exploited widely to date: the RNases H and the double-strand RNases.

ACTIVATION OF RNase H RNase H is a ubiquitous enzyme that degrades the RNA strand of an RNA-DNA duplex. It has been identified in organisms as diverse as viruses and human cells (2). At least two classes of RNase H have been identified in eukaryotic cells. Multiple enzymes with RNase H activity have been observed in prokaryotes (2). Although RNase H is involved in DNA replication, it may play other roles in the cell and is found in the cytoplasm as well as the nucleus (3). However, its concentration in the nucleus is thought to be greater, and some of the enzyme found in cytoplasmic preparations may be due to nuclear leakage. Recently, a knockout of the RNase H1 gene was shown to be embryonically lethal and to inhibit mitochondrial DNA synthesis (4).

The precise recognition elements for RNase H are not known. However, oligonucleotides with DNA-like properties as short as tetramers can activate RNase H (5). Changes in the sugar influence RNase H activation, since sugar modifications that result in RNA-like oligonucleotides, e.g., 2′-fluoro or 2′-methoxy, do not appear to serve as substrates for RNase H (6, 7). Alterations in the orientation of the sugar to the base can also affect RNase H activation, as α-oligonucleotides are unable to induce RNase H or may require parallel annealing (8, 9). Additionally, backbone modifications influence the ability of oligonucleotides to activate RNase H. Methylphosphonates do not activate it (10, 11), whereas phosphorothioates are excellent substrates (12–14). In addition, chimeric molecules have been studied as oligonucleotides that bind to RNA and activate RNase H (15, 16). For example, oligonucleotides comprising wings of 2′-methoxy phosphonates and a five-base gap of deoxyoligonucleotides bind to their target RNA and activate RNase H. Furthermore, a single ribonucleotide in a sequence of deoxyribonucleotides was sufficient to serve as a substrate for RNase H when bound to its complementary deoxyoligonucleotide (17).

It is possible to take advantage of chimeric oligonucleotides designed to activate RNase H, with greater affinity for their RNA receptors, and to enhance specificity (18, 19). In one study, RNase H–mediated cleavage of target transcript was much more selective when deoxyoligonucleotides consisting of methylphosphonate deoxyoligonucleotide wings and phosphodiester gaps were compared to full phosphodiester oligonucleotides (19).

Given the emerging role of chimeric oligonucleotides with modifications in the 3′ and 5′ wings designed to enhance affinity for the target RNA and nuclease stability, and with a DNA-type gap to serve as a substrate for RNase H, studies focused on understanding the effects of various modifications on the efficiency of the enzyme(s) are also important. In one such study on *E. coli* RNase H the enzyme displayed minimal sequence specificity and was processive. When a chimeric

oligonucleotide with 2′-modified sugars in the wings was hybridized to the RNA, the initial site of cleavage was the nucleotide adjacent to the methoxy-deoxy junction closest to the 3′ end of the RNA substrate. The initial rate of cleavage increased as the DNA gap increased, and the enzyme was considerably less efficient against an RNA target duplexed with a chimeric antisense oligonucleotide than a full DNA-type oligonucleotide (20).

Subsequent studies evaluated the interactions of antisense oligonucleotides with structured and unstructured targets, and detailed the impacts of these interactions on RNase H (21). Using a series of noncleavable substrates and Michaelis-Menten analyses, we evaluated both binding and cleavage and showed that, in fact, *E. coli* RNase H1 is a double-strand RNA binding protein. The Kd for the RNA duplex was 1.6 μM; the Kd for a DNA duplex was 176 μM; and the Kd for single-strand DNA was 942 μM. In contrast, the enzyme could only cleave RNA in an RNA-DNA duplex. Any 2′ modification in the antisense drug at the cleavage site inhibited cleavage, but significant charge reduction and 2′ modifications were tolerated at the binding site. Finally, placing a positive charge (e.g., 2′-propoxyamine) in the antisense drug reduced affinity and cleavage. We also examined the effects of antisense oligonucleotide-induced RNA structures on the activity of *E. coli* RNase H1 (21). Any structure in the duplex substrate had a significant negative effect on the cleavage rate. Further, cleavage of selected sites was inhibited entirely, and this was explained by steric hindrance imposed by the RNA loop traversing either the minor or major grooves of the heteroduplex.

Recently, both human RNase H genes have been cloned and expressed (22, 23). The type 1 enzyme is a 286–amino acid protein with a calculated mass of 32 kDa. The enzyme is encoded by a single gene that is at least 10 kb long and expressed ubiquitously in human cells and tissues. The amino acid sequence of human RNase H1 displays strong homology with RNase H1 from yeast (21.8% amino acid identity), chicken (59%), *Escherichia coli* (33.6%), and mouse (84.3%). The type 1 enzymes are all small proteins (<40 kDa) and their estimated p*I* values are all 8, 0.7 and greater. The amino acid resides in *E. coli* RNase H1, which is thought to be involved in the Mg^{2+} binding site, catalytic center, and substrate binding region (24–26). The human RNase H2 enzyme is a 299–amino acid protein with a calculated mass of 33.4 kDa and is also ubiquitously expressed in human cells and tissues (22; Wu, unpublished data). Human RNase H2 shares strong amino acid sequence homology with RNase H2 from *Caenorhabditis elegans* (45.5% amino acid identity), yeast (25.7%), and *E. coli* (14.4%). Unlike the RNase H1 isotype, the type 2 enzyme is an acidic protein exhibiting a p*I* of 4.94.

The properties of the cloned and expressed human RNase H1 have been characterized (27). The activity of the type 1 enzyme is Mg^{2+}-dependent and inhibited by Mn^{2+} and the sulfhydryl blocking agent *N*-ethylmaleimide. Human RNase H1 was also inhibited by increasing ionic strength, with optimal activity for both KCl and NaCl observed at 10–20 m*M*. The enzyme exhibited a bell-shaped response to divalent cations and pH; the optimum conditions for catalysis were 1 m*M* Mg^{2+} and pH 7 to 8. The protein was shown to be reversibly denatured under the

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ACTIVATION OF DOUBLE-STRAND RNases By using phosphorothioate oligonucleotides with 2′-modified wings and a ribonucleotide center, we have shown that mammalian cells contain enzymes that can cleave double-strand RNAs (29). This was important because it added to the repertoire of intracellular enzymes that may be used to cleave target RNAs, and because chimeric oligonucleotides with 2′-modified wings and oligoribonucleotide gaps have higher affinity for RNA targets than chimeras with oligodeoxynucleotide gaps. More recently, the double-strand RNase mechanism, RNAi or siRNA, has generated excitement (30–40).

Figure 1 compares RNase and RNAi mechanisms. At Isis Pharmaceuticals, we exploited double-strand RNases by chemically modifying single-strand RNA to stabilize it instead of using double-strand RNA. Recently, several comparisons of RNase H to RNAi as approaches for gene functionalization have been reported (41).

Double-strand RNases, especially the processes involved in RNAi, have provided new opportunities to identify new mechanisms for antisense that may differ from RNase H.

CHARACTERISTICS OF PHOSPHOROTHIOATE OLIGONUCLEOTIDES

Of the first-generation oligonucleotide analogs, the phosphorothioate class is the best known and has yielded the broadest range of activities. Phosphorothioate oligonucleotides were first synthesized in 1969, when a poly (rIrC) phosphorothioate was synthesized. This modification clearly achieves the objective of increased nuclease stability. In this class of oligonucleotides, one of the oxygen atoms in the phosphate group is replaced with a sulfur. The resulting compound is negatively charged, is chiral at each phosphorothioate phosphodiester, and is much more resistant to nucleases than the parent phosphorothioate (42).

Interactions with Proteins

Phosphorothioate oligonucleotides bind to proteins. Their interactions with proteins can be divided into nonspecific, sequence-specific, and structure-specific binding events, each of which may have different characteristics and effects. Nonspecific binding to a wide variety of proteins has been demonstrated. Exemplary

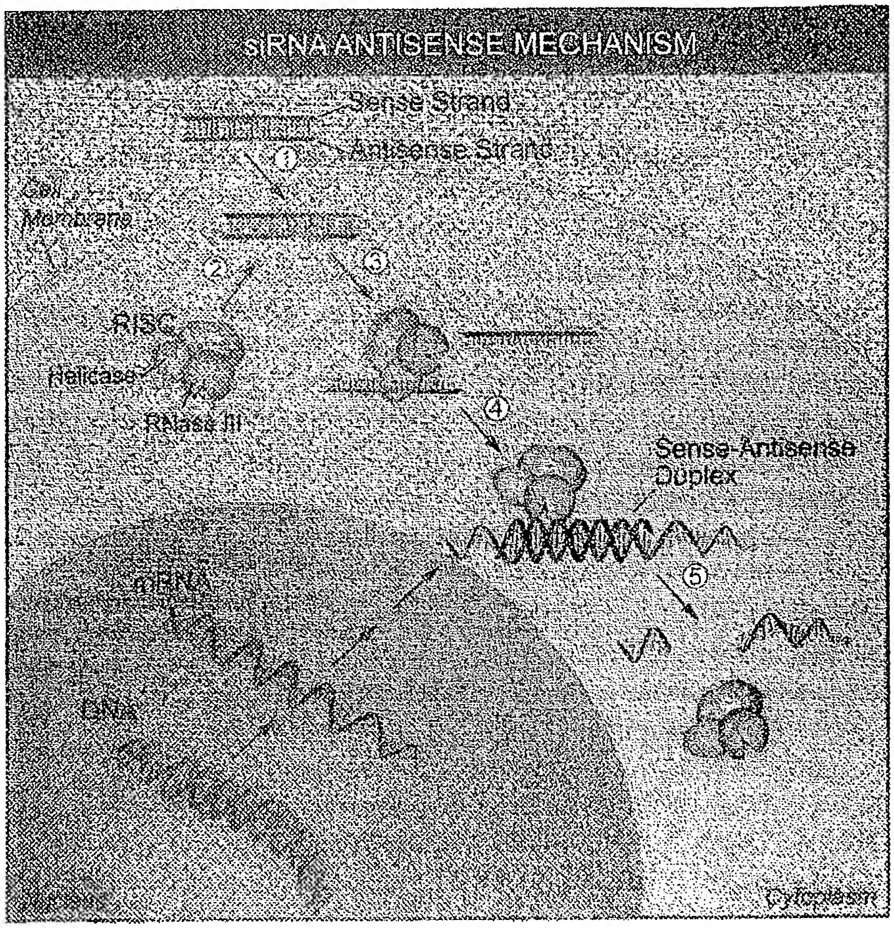
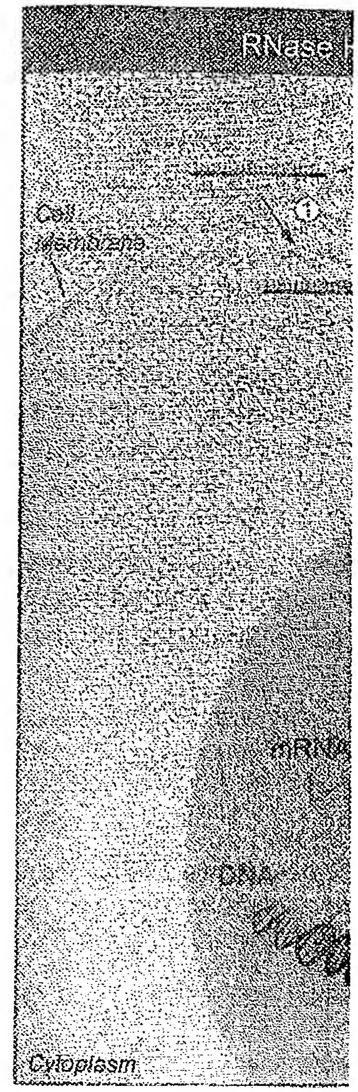


Figure 1a (1) The double-strand oligonucleotide passes through the cell membrane and enters the cytoplasm. (2) The helicase separates the sense and antisense strands of the oligonucleotide. (3) The RISC complex, an endogenous conglomerate of functional components, associates with the antisense oligonucleotide. (4) The antisense strand of the oligonucleotide binds (hybridization) to the target mRNA, forming a sense-antisense duplex. (5) The nuclease component of RISC is an endogenous nuclease that degrades the target mRNA. This inhibits target mRNA expression.

of this type of binding is the interaction of phosphorothioate oligonucleotides with serum albumin. The affinity of such interactions is low. The Kd for albumin is ~200 μM, in a similar range with aspirin or penicillin (43, 44). Binding to serum protein, including albumin, prevents rapid clearance by glomerular filtration, thus providing the opportunity for these drugs to distribute to peripheral tissues. Phosphorothioates interact with many different proteins and these interactions play important roles in the distribution, clearance, and toxicological profiles of the



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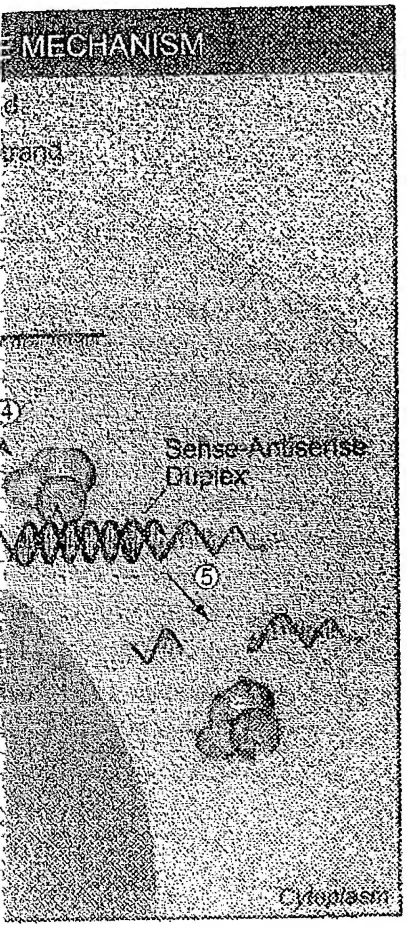

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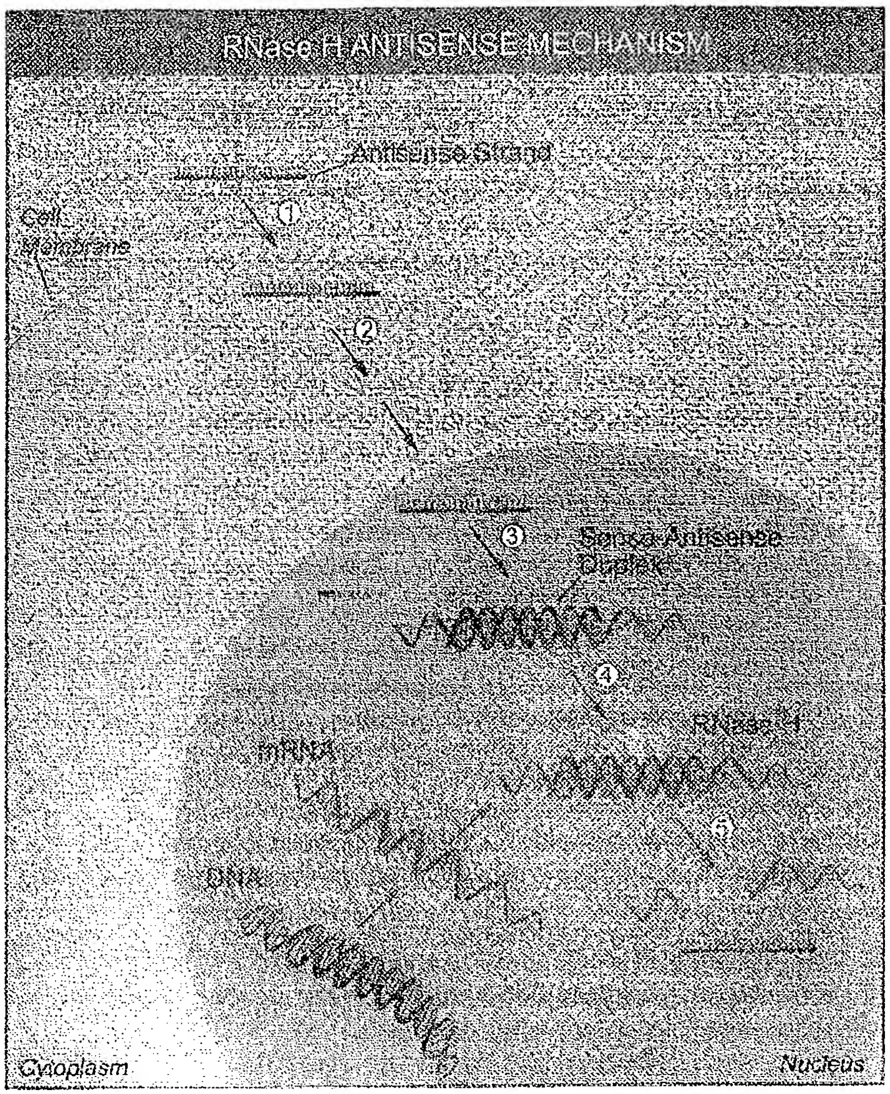

Figure 1b (1) The single-strand DNA oligonucleotide passes through the cell membrane and enters the cytoplasm. (2) The oligonucleotide enters the nucleus. (3) The oligonucleotide binds (hybridizes) to the target mRNA, forming a sense-antisense duplex. (4) The formation of the duplex initiates the recruitment of the RNase H enzyme, an endogenous nuclease. (5) RNase H degrades the target mRNA, inhibiting target mRNA expression. The oligonucleotide moves on and binds (hybridizes) to another target RNA.

drugs (44–57). In this regard, antisense drugs do not significantly differ from most drugs and the summation of these interactions is measured in the therapeutic index that the first-generation ASOs display.

Nuclease Stability

The principal metabolic pathway for oligonucleotides is cleavage via endo- and exonucleases. Phosphorothioate oligonucleotides, though quite stable to various nucleases, are competitive inhibitors of nucleases (50, 58–61). Consequently, the stability of phosphorothioate oligonucleotides to nucleases is probably a bit less than initially thought, since early studies used concentrations of oligonucleotides that were high enough to inhibit nucleases. Similarly, phosphorothioate oligonucleotides are degraded slowly by cells in tissue culture with a half-life of 12–24 h and are slowly metabolized in animals (60, 62, 63). The pattern of metabolites suggests primarily exonuclease activity with perhaps modest contributions by endonucleases. However, several lines of evidence suggest that, in many cells and tissues, endonucleases are important in the metabolism of oligonucleotides. For example, 3′- and 5′-modified oligonucleotides with phosphodiester backbones are degraded relatively rapidly in cells and after administration to animals (64, 65). Thus, stability-enhancing strategies in which oligonucleotides are modified after only the 3′ and 5′ terminus have failed.

In Vitro Cellular Uptake

Phosphorothioate oligonucleotides are taken up by a wide range of cells in vitro (50, 59, 66–68). In fact, uptake of phosphorothioate oligonucleotides into a prokaryote, *Vibrio parahaemoyticus*, has been reported, as has uptake into *Schistosoma mansoni* (69, 70). Uptake is time- and temperature-dependent. It is also influenced by cell type, cell-culture conditions, media and sequence, and length of the oligonucleotide (59). No correlation has been found between uptake and the lineage of cells, whether the cells are transformed or virally infected. Nor are the factors causing differences in uptake of different sequences of oligonucleotide understood. Although several studies have suggested that receptor-mediated endocytosis may be a significant mechanism of cellular uptake, the data are not yet conclusive (71).

Numerous studies have shown that phosphorothioate oligonucleotides distribute broadly in most cells once taken up (59, 72). Again, however, significant differences in subcellular distribution between various types of cells are noted.

Cationic lipids have been used to enhance uptake of phosphorothioate oligonucleotides in cells that take up little oligonucleotide in vitro (73–75). Here too there are substantial variations from cell type to cell type. Other approaches to enhanced intracellular uptake in vitro have included streptolysin D treatment of cells and the use of dextran sulfate and other liposome formulations, as well as physical means such as microinjections (59, 76, 77).

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In Vivo Pharmacokinetics

Phosphorothioate oligonucleotides bind to serum albumin and α-2 macroglobulin. The apparent affinity for albumin is quite low (200–400 μM), comparable to the low-affinity binding observed for a number of drugs, including aspirin and penicillin (62–64). Serum protein binding, therefore, provides a repository for these drugs and prevents rapid renal excretion. Because serum protein binding is saturable, at higher doses, intact oligomer may be found in urine (52, 78). Our studies suggest that in rats, oligonucleotides administered intravenously at doses of 15–20 mg/kg saturate the serum protein binding capacity (79).

Phosphorothioate oligonucleotides are rapidly and extensively absorbed after parenteral administration. In rats, after an intradermal dose 3.6 mg/kg of ^{14}C-ISIS 2105, a 20-mer phosphorothioate, ~70% of the dose was absorbed within 4 h and total systemic bioavailability was in excess of 90% (80). Absorption of ISIS 2105 was similar after intradermal injection in humans (81). Subcutaneous administration to rats and monkeys results in somewhat lower bioavailability and greater distribution to lymph nodes, as would be expected (72).

Distribution of phosphorothioate oligonucleotides from blood after absorption or intravenous (IV) administration is extremely rapid. Distribution half-lives of less than 1 h are reported (52, 62, 78, 80). Blood and plasma clearance is multi-exponential, with a terminal elimination half-life of 40–60 h in all species except humans. In humans the terminal elimination half-life may be somewhat longer (82).

Phosphorothioates distribute broadly to all peripheral tissues. Liver, kidney, bone marrow, skeletal muscle, and skin accumulate the highest percentage of a dose, but other tissues contain small quantities of drug (80, 83). No evidence of significant penetration of the blood-brain barrier has been reported. The rates of incorporation and clearance from tissues vary among organs, with the liver accumulating drug most rapidly (20% of a dose within 1–2 h). Similarly, elimination of drug from liver is rapid compared to many other tissues; the terminal half-life from liver is 62 h, whereas from renal medulla it is 156 h. The distribution into the kidney has been studied more extensively. Phosphorothioates were found in Bowman's capsule, in the proximal convoluted tubule, in the bush-border membrane, and within renal tubular epithelial cells (84). The data suggested that the oligonucleotides are filtered by the glomerulus and then reabsorbed by the proximal convoluted tubule epithelial cells. Reabsorption might be mediated by interactions with specific proteins in the bush-border membranes (84). In addition, oligonucleotide is accumulated in a nonfiltering kidney, suggesting that there is uptake from the basal side also.

Clearance of phosphorothioate oligonucleotides is due primarily to metabolism (62, 78, 80). Metabolism is mediated by exo- and endonucleases that result in shorter oligonucleotides and, ultimately, nucleosides that are degraded by normal metabolic pathways. Base excision or modification are theoretically possible, although no direct evidence of them has been reported. One study found, but did

not fully characterize, a larger-molecular-weight radioactive material in urine (52). Clearly, the potential for conjugation reactions and extension of oligonucleotides via these drugs serving as primers for polymerases must be explored in more detail. In a very thorough study, 20 nucleotide phosphodiester and phosphorothioate oligonucleotides were administered intravenously to mice at a dose of 6 mg/kg. The oligonucleotides were internally labeled with $^3H\text{-}CH_3$ by methylation of an internal deoxycytidine residue using HhaI methylase and S-(^{3}H) adenosyl methionine (85). The observed pharmacokinetic properties were consistent with our results as described above. Additionally, autoradiographic analyses showed drug in renal cortical cells (84, 85).

A study that carefully measured intact drug by capillary gel electrophoresis determined the pharmacokinetics of Alicaforsen (a 20-mer phosphorothioate oligodeoxynucleotide) after a 2-h infusion. Doses from 0.06 mg/kg to 2.0 mg/kg were studied. Peak plasma concentrations increased linearly with dose; the 2-mg/kg dose resulted in peak plasma concentrations of intact drug of ~9.5 μg/ml. Clearance from plasma, however, was dose-dependent. The clearance of the 2-mg/kg dose was 1.28 ml min^{-1} kg^{-1}, whereas that of 0.5 mg/kg was 2.07 ml min^{-1} kg^{-1}. Essentially no intact drug was found in urine.

In addition to the pharmacologic effects of phosphorothioate oligonucleotides on animals and humans, several other lines of evidence show that these drugs enter cells in organs. Autoradiographic, fluorescent, and immunohistochemical approaches have shown that these drugs are localized in endopromal convoluted tubular cells, various bone marrow cells, skin cells, and liver cells (83, 84, 86).

Perhaps more compelling and of more long-term value is a study showing the distribution of phosphorothioate oligonucleotides in the liver of rats treated intravenously at various doses (87). The kinetics and extent of the accumulation into Kuppfer, endothelial, and hepatocyte cell populations varied, and as doses were increased, the distribution changed. Moreover, subcellular distribution was significant and somewhat dependent on the cell type.

In summary, pharmacokinetic studies of several phosphorothioates demonstrate that they are well absorbed from parenteral sites, distribute broadly to many peripheral tissues, do not cross the blood-brain barrier, and are eliminated primarily by nuclease metabolism. In short, once-daily or every-other-day systemic dosing is feasible. Although the similarities between oligonucleotides of different sequences far outweigh the differences, additional studies are required to see whether sequence exerts subtle effects on the pharmacokinetic profile of this class of drugs.

Modes of Administration

Phosphorothioate oligodeoxynucleotides are attractive for inhalation delivery to the lung and upper airway (71, 88, 89). Target reduction in the lung has been demonstrated, and these drugs have been shown to distribute broadly to all cell types in the lung after aerosol administration. Further, these drugs are well tolerated at doses up to 12 mg/kg (71).

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PHARMACOLOGIC PRO

Molecular Pharmacology

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Phosphorothioate oligonucleotides in a simple cream formulation penetrate normal mouse, pig, and human skin, and they penetrate and accumulate in human psoriatic skin grown on nude mice 6.5.2a, 6.5.2b. Further, a phase IIa study in patients with plaque psoriasis showed that Alicaforsen, an inhibitor of intercellular adhesion molecule 1 (ICAM-1), accumulated throughout the dermis and epidermis after topical administration and resulted in a positive trend in the primary endpoint, induration (Kruger, unpublished observations). Studies have also demonstrated reduction of targets such as ICAM-1, B71, and B72 (for review see 90).

Alicaforsen has also been administered by enema and shown to be effective in treating ulcerative colitis (91).

PHARMACOLOGIC PROPERTIES

Molecular Pharmacology

Although there are multiple mechanisms by which an oligonucleotide may terminate the activity of an RNA species to which it binds, evidence has been reported for only three of these mechanisms. Antisense oligonucleotides have been reported to inhibit RNA splicing, affect translation of mRNA, and induce degradation of RNA by RNase H (92–94). Without question, the mechanism that has resulted in the most potent compounds and is best understood is RNase H activation. To serve as a substrate for RNase H, a duplex between RNA and "DNA-like" oligonucleotide is necessary. Specifically required are a charged phosphate and a sugar moiety in the oligonucleotide that induces a duplex conformation equivalent to that of a DNA-RNA duplex (95). Thus, phosphorothioate oligodeoxynucleotides are expected to induce RNase H-mediated cleavage of the RNA when bound. Many chemical approaches that enhance the affinity of an oligonucleotide for RNA result in duplexes that are no longer substrates for RNase H.

Selection of sites for induction of optimal antisense activity in an RNA molecule is dependent on the terminating mechanism and influenced by the chemical class of the oligonucleotide. Each RNA appears to display unique patterns of sites of sensitivity. Within the phosphorothioate oligodeoxynucleotide chemical class, antisense activity can vary from undetectable to 100% by shifting an oligonucleotide by just a few bases in the RNA target (92, 96, 97). Despite significant progress in developing general rules that help define potentially optimal sites in RNA species, to a large extent, this remains an empirical process that must be performed for each RNA target and every new chemical class of oligonucleotides.

Phosphorothioates have also shown effects inconsistent with the antisense mechanism for which they were designed. Some of these effects are due to sequence or are structure-specific. Others are due to nonspecific interactions with proteins. These effects are particularly prominent in in vitro tests for antiviral activity, where high concentrations of cells, viruses, and oligonucleotides are often coincubated (98, 99). Human immune deficiency virus (HIV) is particularly problematic because many oligonucleotides bind to the gp120 protein (56). However,

the potential for confusion arising from the misattribution of an activity to an antisense mechanism when, in fact, it is due to nonantisense effects is certainly not limited to antiviral or in vitro tests (100–102). These data urge caution and argue for careful dose-response curves, direct analyses of target protein or RNA, and inclusion of appropriate controls before drawing conclusions concerning the mechanisms of action of oligonucleotide-based drugs. In addition to protein interactions, other factors, such as overrepresented sequences of RNA and unusual structures that may be adopted by oligonucleotides, can contribute to unexpected results (56).

Given the variability in cellular uptake of oligonucleotides, the variability in potency as a function of binding site in an RNA target, and the potential nonantisense activities of oligonucleotides, careful evaluation of dose-response curves and clear demonstration of the antisense mechanism are required before drawing conclusions from in vitro experiments. Nevertheless, numerous well-controlled studies have conclusively demonstrated antisense activity against a variety of targets (12, 51, 96, 103, 104).

In Vivo Pharmacologic Activities

In vivo activities of phosphorothioate oligonucleotides after both local and systemic administration have been reported (for review see 105). Here I review only the few reports that provide sufficient data to support a relatively firm conclusion about mechanism of action.

Local effects have been reported for phosphorothioate and methylphosphonate oligonucleotides. In one study (106), a phosphorothioate oligonucleotide designed to inhibit c-*myb* production and applied locally inhibited intimal accumulation in the rat carotid artery. A Northern blot analysis showed a significant reduction in c-*myb* RNA in animals treated with the antisense compound, a control oligonucleotide had no effect. A study attributed the effects of the oligonucleotide to a nonantisense mechanism (101). However, only one dose level was studied, so definitive conclusions are not yet possible. Similar effects were reported for phosphorothioate oligodeoxynucleotides designed to inhibit cyclin-dependent kinases (CDC-2 and CDK-2). Again, the antisense oligonucleotide inhibited intimal thickening and cyclin-dependent kinase activity, whereas a control oligonucleotide had no effect (107). Additionally, local administration of a phosphorothioate oligonucleotide designed to inhibit N-*myc* reduced N-*myc* expression and slowed the growth of a subcutaneously transplanted human tumor in nude mice (108, 109).

Antisense oligonucleotides administered intraventricularly have induced various effects in the central nervous system. Intraventricular injection of antisense oligonucleotides to neuropeptide-y-y1 receptors reduced the density of the receptors and caused anxious behavior (109). Similarly, an antisense oligonucleotide designed to bind to NMDA-R1 receptor channel RNA inhibited the synthesis of these channels and reduced the volume of focal ischemia produced by occlusion of the middle cerebral artery in rats (110).

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In a series of well-controlled studies, intraventricular administration of antisense oligonucleotides selectively inhibited dopamine type-2 receptor expression, dopamine type-2 receptor RNA levels, and behavioral effects in animals with chemical lesions. Controls included randomized oligonucleotides and the observation that no effects were observed on dopamine type-1 receptor or RNA levels (16–18). This laboratory also reported the selective reduction of dopamine type-1 receptor and RNA levels with the appropriate oligonucleotide (111). Numerous other targets have been inhibited after central nervous system administration as well (for review see 112).

In rabbits, aerosol administration of an antisense phosphorothioate oligodeoxynucleotide designed to inhibit the production of adenosine A_1 receptor reduced receptor numbers in the airway smooth muscle and inhibited adenosine, house dust-mite allergen, and histamine-induced bronchoconstriction (113). Neither a control nor an oligonucleotide complementary to bradykinin B_2 receptors reduced the density of adenosine A_1 receptors, but oligonucleotides complementary to bradykin in B_2 receptor mRNA did.

In addition to local and regional effects of antisense oligonucleotides, well-controlled studies have demonstrated systemic effects of phosphorothioate oligodeoxynucleotides. Expression of interleukin-1 in mice was inhibited by systemic administration of antisense oligonucleotides (113). Oligonucleotides to the NF-κB p65 subunit administered intraperitoneally at 40 mg/kg every 3 days slowed tumor growth in mice transgenic for the human T-cell leukemia viruses (21). Another in vivo tumor model yielded similar results after either prolonged subcutaneous infusion or intermittent subcutaneous injection of other antisense oligonucleotides (114).

Several recent reports further explore phosphorothioate oligonucleotides as antitumor agents in mice. In one study, a phosphorothioate oligonucleotide directed to inhibit the bcr-abl oncogene was administered intravenously at a dose of 1 mg/day for 9 days to immunodeficient mice injected with human leukemic cells. The drug inhibited the development of leukemic colonies in the mice and selectively reduced bcr-abl RNA levels in peripheral blood lymphocytes, spleen, bone marrow, liver, lungs, and brain (115). However, the effects on the RNA levels could have been secondary to effects on the growth of various cell types. In the second study, a phosphorothioate oligonucleotide antisense to the protooncogene myb inhibited the growth of human melanoma in mice. Again, myb mRNA levels appeared to be selectively reduced (116).

A number of studies have directly examined target RNA levels, target protein levels, and pharmacologic effects using a wide range of control oligonucleotides, and have examined the effects on closely related isotypes. Single and chronic daily administration of a phosphorothioate oligonucleotide designed to inhibit mouse protein kinase C-α (PKC-α) selectively inhibited expression of PKC-α RNA in mouse liver without effects on any other isotype. The effects lasted at least 24 h after a dose, and a clear dose-response curve was observed, with an intraperitoneal dose of 10–15 mg/kg reducing PKC-α RNA levels in liver by 50% after 24 h (117).

IV administration of a phosphorothioate oligonucleotide designed to inhibit human PKC-α expression selectively inhibited expression of PKC-α RNA and PKC-α protein in human tumor cell lines implanted subcutaneously in nude mice (118). In these studies, effects on RNA and protein levels were highly specific and observed at doses lower than 6 mg/kg. A large number of control oligonucleotides failed to show activity.

In a similar series of studies, Monia et al. demonstrated highly specific loss of human *c-raf* kinase RNA in human tumor xenografts and antitumor activity that correlated with the loss of RNA (119, 120).

Finally, a single injection of a phosphorothioate oligonucleotide designed to inhibit c-AMP-dependent protein kinase type 1 was reported to selectively reduce RNA and protein levels in human tumor xenografts and to reduce tumor growth (121). This growing body of evidence indicates that phosphorothioate oligonucleotides can induce potent systemic and local effects in vivo. More importantly, there have been several studies with sufficient controls and direct observation of target RNA and protein levels to suggest highly specific effects that are difficult to explain via any mechanism other than antisense. As would be expected, the potency of these effects varies with the target, the organ, and the endpoint measured, as well as the route of administration and the time after a dose when the effect is measured.

In conclusion, although in vivo activity data must be interpreted cautiously, and it is clearly necessary to include a range of controls and to evaluate effects on target RNA and protein levels and control RNA and protein levels directly, some effects have been observed in animals that are most likely primarily due to an antisense mechanism.

CLINICAL ACTIVITIES

A supplemental table (see Supplemental Material at http://www.annualreviews.org/supmat/supmat.asp) provides a comprehensive summary of all the clinical reports on antisense drugs.

Vitravene™, a phosphorothioate oligonucleotide designed to inhibit cytomegalovirus-induced retinitis, has been shown to be safe and effective in the treatment of this disease after intravitreal injection and has been approved by regulatory agencies in the United States, Europe, and South America (for review see 122).

Alicaforsen

Alicaforsen (Isis Pharmaceuticals, Carlsbad, CA) is a phosphorothioate oligonucleotide inhibitor of human ICAM-1. It reduces ICAM-1 levels in several organs in various animals and has potent antiinflammatory effects (for review see 123).

A number of clinical trials have evaluated Alicaforsen in patients with inflammatory diseases (for review see 124). The drug was evaluated in a randomized

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placebo-controlled phase IIa trial in 43 patients with moderate to severe rheumatoid arthritis (125). The effects of 0.5, 1.0, and 2.0 mg/kg IV three times weekly for one month were compared to placebo. The 0.5 mg/kg and 2.0 mg/kg doses resulted in prolonged improvement in rheumatoid arthritis activity. All doses were extremely well tolerated.

Alicaforsen was evaluated as a potential therapeutic for patients with psoriasis (126). This study compared IV and topical routes of administration. The dosing for the IV study was equivalent to that used for the rheumatoid arthritis study, but only 17 patients were evaluated. Again, all doses were well tolerated. At the end of the treatment period, ~30% reduction in psoriasis activity index score was reported (126). Additionally, several concentrations of Alicaforsen, applied topically in a simple cream formulation, were evaluated in patients with moderate plaque psoriasis. The drug was applied once a day for 3 months, and patients were followed for a total of 6 months. In approximately half the patients, skin biopsies were taken. Alicaforsen achieved very high dermal and epidermal concentrations that increased as the concentration in the cream was increased. At 2% and 4% concentrations, the creams produced dermal concentrations similar to those at which pharmacologic effects were observed in animals. In regard to the primary endpoint, induration, there was trend ($p = 0.053$) in favor of increasing Alicaforsen concentrations. Again the drug was very well tolerated.

Alicaforsen has been evaluated most extensively in Crohn's disease. In the initial placebo-controlled study in steroid-dependent patients, Alicaforsen reduced symptoms, steroid use, and ICAM-1 levels in the small intestines of patients treated and evaluated with serial colonoscopies. The duration of effect after a month of dosing was in excess of 6 months (127). In a subsequent 300-patient trial, in which patients were treated with placebo or 2 mg/kg every other day for 2 or 4 weeks, again the drug was extremely well tolerated. A population pharmacokinetic study demonstrated that heavier patients and women achieved greater exposures to the drug, and in the patients in the upper two quartiles of drug exposure, the drug produced a statistically significant increase in complete remissions compared to placebo (128). More recently, a higher-dose phase II study in Crohn's showed that the higher doses were well tolerated and active (129). Additional phase III trials at higher doses are in progress (91).

In addition, Alicaforsen has been administered as a retention enema to patients with ulcerative colitis. A randomized double-blind trial demonstrated a clear dose-response relationship, and the top dose was extremely active. All treated patients responded. The mean reduction in the ulcerative colitis disease index was 73% versus 23% for placebo ($p = 0.004$). All patients experienced prolonged benefit with no relapses for 6 months after 1 month of dosing (91).

In a related syndrome, pouchitis, Alicaforsen has shown considerable promise. Pouchitis occurs in patients with ulcerative colitis who have undergone resection of their colon, reanastomosis of the small bowel to the rectum, and construction of a pseudocolon or pouch. A significant fraction of these patients developed inflammation in the pouch. Alicaforsen provided a highly statistically significant

benefit in these patients (91). Thus, evidence indicates that Alicaforsen is active in inflammatory disease when administered intravenously, topically, or by enema.

Affinitak

Affinitak (Isis Pharmaceuticals, Carlsbad, CA) is a phosphorothioate oligodeoxynucleotide inhibitor of protein kinase Cα (for review see 130). This drug has been evaluated as a single agent in a variety of solid tumors, using a range of dose schedules at doses as high as 30 mg/kg/day, and has been well tolerated. The supplemental table shows results in patients with advanced chemoresistant malignancies. Affinitak has also been evaluated in combination with various chemotherapeutic agents in a number of solid tumors. In a phase I/II study in 53 patients with non–small-cell carcinoma of the lung, Affinitak was associated with a substantial improvement in survival (16 months) compared to historical controls (131). Based on these data, a large phase III program was designed.

The first study in the phase III program was a 616-patient randomized trial in patients with stage IIIb or IV non–small-cell carcinoma of the lung. Patients were randomized to receive either standard doses of carboplatinum-taxol or carboplatinum-taxol plus Affinitak. Affinitak was administered via an indwelling central venous catheter as a continuous IV infusion of 2 mg/kg/day for 2 weeks out of every 3-week cycle. Six cycles were considered a complete course of therapy.

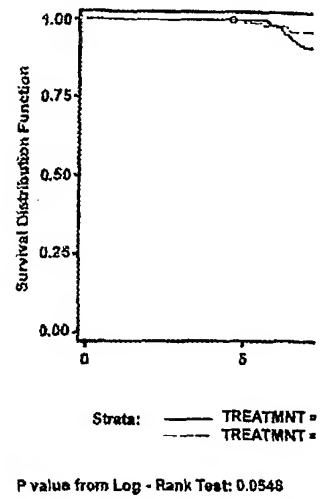
Although the Affinitak arm did not achieve the primary statistical endpoint, substantial evidence suggested activity (132). Table 1 analyzes survival as a function of whether patients were able to complete all six cycles of therapy. Note that survival improved in both the "completers" and "noncompleters." Figure 2 shows the survival of completers in the two groups. These analyses suggest that the survival advantage afforded by Affinitak was total-dose–related and potentially significant.

Affinitak, when added to carboplatinum and taxol, was well tolerated. Aside from the expected central-line complications, the only clinically meaningful observation was an increase in mild to moderate thrombocytopenia.

A second study, in which Affinitak is added to gemcitabine and cisplatinum in patients with non–small-cell carcinoma of the lung, is in progress and results are expected in 2004.

TABLE 1 Summary of major survival parameters

	Log Rank Test				
	Exp.		Control		
	Median	n	Median	N	p Value
ITT overall	10.0	309	9.7	307	0.8054
Completers	17.3	114	14.4	142	0.0548
Noncompleters	6.5	195	5.2	165	0.6406



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Control		
Median	N	p Value
9.7	307	0.8054
14.4	142	0.0548
5.2	165	0.6406

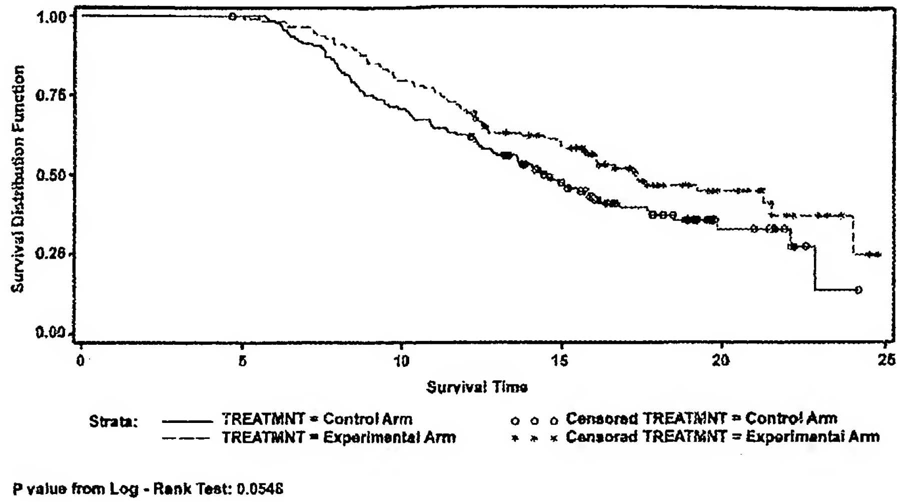

Figure 2 Patients who completed therapy appeared to experience Affinitak-associated survival benefit relative to controls.

GeneSense

GeneSense is a phosphorothioate oligodeoxynucleotide designed to inhibit BCL_2. In a non-Hodgkin's lymphoma trial, this single agent reduced BCL_2 levels in peripheral blood cells (133). GeneSense is also being studied in combination with chemotherapeutic agents. A phase I study with decarbazine in patients with malignant melanoma reported a 21% response rate in 14 patients (134). This drug is being evaluated in other combinations for other malignant diseases.

ISIS 2503

ISIS 2503 is a phosphorothioate oligodeoxynucleotide that inhibits *Ha-ras*. It is currently completing phase II single-agent and combination studies in patients with various solid malignancies (for review see 130). It is well tolerated and shows preliminary suggestions of activity (see supplemental table at http://www.annualreviews.org/supmat/supmat.asp).

More recently, ISIS 2503 was combined with gemcitabine in a 40-patient phase II study in patients with pancreatic cancer. The combination was well tolerated and displayed potential benefit.

ISIS 5132

ISIS 5231 is a phosphorothioate oligodeoxynucleotide inhibitor of *c-raf* kinase. In phase I studies, this drug reduced *c-raf* kinase levels in peripheral blood cells and displayed activity in patients with ovarian cancer (135). The phase II evaluation, however, failed to show meaningful activity for the drug (for review see 130).

TOXICOLOGY

Genotoxicity

Concerns about the genotoxicity of phosphorothioate oligodeoxynucleotides cannot be dismissed; our understanding of the basic mechanisms of action is limited, little in vitro testing has been performed, and no data from long-term studies of oligonucleotides are available. We have performed mutagenicity studies on five phosphorothioate oligonucleotides [Alicaforsen, ISIS 2105, ISIS 5132, ISIS 14803, and Vitravene™ (Isis Pharmaceuticals, Carlsbad, CA)] and found them to be nonmutagenic at all concentrations studied (82, 136).

Two mechanisms of genotoxicity that may be unique to oligonucleotides have been considered. One possibility is that an oligonucleotide analog could be integrated into the genome and produce mutagenic events. Although such integration is conceivable, it is likely to be extremely rare. For most viruses, viral DNA integration is itself a rare event and, of course, viruses have evolved specialized enzyme-mediated mechanisms to achieve integration. Moreover, our preliminary studies have shown that phosphorothioate oligodeoxynucleotides are generally poor substrates for DNA polymerases, and enzymes such as integrases, gyrases, and topoisomerases (which have obligate DNA cleavage as intermediate steps in their enzymatic processes) are unlikely to accept these compounds as substrates. Consequently, it would seem that the risk of genotoxicity due to genomic integration is no greater than and probably less than that of other potential mechanisms (e.g., alteration of the activity of growth factors, cytokine release, and nonspecific effects on membranes that might trigger arachidonic acid release or inappropriate intracellular signaling). Presumably, new analogs that deviate significantly more from natural DNA would be even less likely to be integrated.

A second genotoxicity concern is that oligonucleotides might be degraded to toxic or carcinogenic metabolites. However, metabolism of phosphorothioate oligodeoxynucleotides by base excision would release normal bases, which presumably would be nongenotoxic. Similarly, oxidation of the phosphorothioate backbone to the natural phosphodiester structure would also yield nonmutagenic (and probably nontoxic) metabolites. Finally, it is possible that phosphorothioate bonds could be hydrolyzed slowly, releasing nucleoside phosphorothioates that presumably would be rapidly oxidized to natural (nontoxic) nucleoside phosphates. However, oligonucleotides with modified bases and/or backbones may pose different risks.

Acute and Transient Toxicities In Vivo

COMPLEMENT ACTIVATION Rapid infusion of phosphorothioate oligodeoxynucleotides in nonhuman primates can result in cardiovascular collapse (137–139). Complement activation is necessary but not sufficient to produce the observed cardiovascular effects (for review see 46). The other factors contributing to cardiovascular collapse are not yet fully elucidated. However, it has been suggested

that dosing monkeys tha
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Subchronic Toxicities

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that dosing monkeys that are restrained may exacerbate cardiovascular events. Activation of the complement cascade due to activation of the alternate pathway is insensitive to sequence, but is absolutely related to peak plasma concentration (140). The threshold concentration for activation of complement in the monkey is 40–50 μg/ml phosphorothioate oligodeoxynucleotide, and once the threshold is reached, variable but potentially dangerous levels of complement activation are observed.

Studies in humans have avoided peak plasma concentrations that would induce complement. However, it appears that monkeys are substantially more sensitive to complement activation than humans. A comparison of the effects on complement activation in human versus monkey serum demonstrates a dramatic difference. In monkey serum, phosphorothioate oligodeoxynucleotides activate complement in a concentration-dependent fashion. It human serum, complement activation is actually inhibited at higher concentrations of oligonucleotide (46). This effect is thought to be due to the sensitivity of human serum to inhibition of complement activators. The mechanism of complement activation is currently believed to be an interaction with factor H (46). These effects can be reduced by chemical modifications and formulations that reduce plasma protein binding (46).

INHIBITION OF CLOTTING In all species studies, phosphorothioate oligodeoxynucleotides induce a transient, apparently self-limited, peak plasma concentration–related inhibition of clotting, manifested as an increase in activated partial thromboplastin time (aPTT) (141–144). Increases in aPTT are minimally affected by sequence, but the effects are directly proportional to the length of the phosphorothioate oligodeoxynucleotides (46). The mechanism of aPTT increase seems to be an interaction with the intrinsic tenase complex (145, 146). This complex interaction involves effects on multiple clotting factors, including factors VIIIa, IXa, and X. The effects on clotting are transient, appear to be self-limited, and have not caused bleeding diatheses in animals or humans. Clotting inhibition can be ameliorated by chemical modifications such as 2′-O-(methoxyethyl) (2′-*O*-MOE) substitution (46).

Subchronic Toxicities

IMMUNE STIMULATION In rodents, the most prominent toxicity is immune stimulation. This is also frequently a confounding variable that must be evaluated with regard to the mechanism of action of pharmacologic effects (for review see 136). Subchronic administration of doses as low as 10 mg/kg/day in rodents results in splanomegaly, lymphoid hyperplasia, and diffuse multiorgan mixed mononuclear cell infiltrates (140, 147–149). These effects are reminiscent of stimulator effects observed on isolated splemocytes (150–152). In fact, these in vitro studies are reasonably predictive of the relative potencies of phosphorothioates in moving in vivo immune stimulation. Immune stimulation in rodents is a property common to all phosphorothioate oligodeoxynucleotides, but potency varies substantially as

a function of sequence. Rodent immune stimulatory motifs include palindromic sequences and CG motifs (153, 154).

In primates, immune stimulation is far less prominent. Doses that produce immune stimulation in monkeys have not been identified despite evaluation of numerous oligonucleotides at a variety of doses and schedules (46). At least one factor contributing to this species difference is that the optimal rodent immune stimulator sequence is simple and immune stimulation is induced by all sequences. In contrast, in primates, the optimal sequences are different and more complex (155) and the response is much less promiscuous with regard to sequence. Modifications of the base, sugar, and backbone can reduce immune stimulation in rodents. For example, 5′-methycytosine and 2′-*O*-MOE containing oligonucleotides display substantially reduced potency for immune stimulation in rodents (46).

Other Toxicities

In rodents but not in monkeys, single-cell hepatolyte neurons are occasionally observed and have been related to immunostimulation. In monkeys, transient thrombocytopenia is occasionally observed, perhaps associated with complement activation (for review see 156). Other toxicities in animals are mild and infrequent at therapeutic doses. For example, occasional increases in liver function enzymes are noted, but these occur at high doses and are not associated with histopathological changes (46).

Human Safety

At Isis, we have studied ~10 phosphorothioate oligonucleotides in humans. We have studied antisense drugs administered intravitreally, intradermally, subcutaneously, intravenously, and topically. Vitravene™, an intravitreally administered drug, is commercially available around the world.

After systemic administration, in clinical trials, we have studied more than 2000 patients treated with more than 70,000 doses. IV doses have ranged from 0.5 mg/kg every other day to as much as 30 mg/kg/day as a continuous infusion. Most patients have been treated with multiple doses and many have been treated for multiple months.

COMPLEMENT ACTIVATION With 2-hour infusions at 2 mg/kg, no increases in complement split products were observed in more than 300 patients with inflammatory diseases treated with Alicaforsen, an inhibitor of ICAM-1. Similarly, ISIS 5132, an inhibitor of *c-raf* kinase, was dosed from 0.5–6.0 mg/kg with no meaningful increases in complement split products. Affinitak, a PKCα inhibitor, gave equivalent data and both of these drugs were studied in patients with various malignant diseases. Similar results were observed with all the phosporothioate oligodeoxynucleotides administered on this schedule in a variety of patients (for review see 157, 158). Three phosphorothioate oligodeoxynucleotides—Affinitak, ISIS 5132, and ISIS 2503 (an H-*ras* inhibitor)—have been thoroughly characterized in patients with malignant diseases who were dosed with long-term infusions.

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CYTOKINES At very high
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THE MEDICINAL CHEMIS

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o were dosed with long-term infusions.

When given as 21-day antitumor infusions at doses as high as 10 mg/kg/day, no increases in complement split products were observed (159–161). In contrast, when these drugs were given as 24-h infusions, significant increases in complement split products occurred at doses of 18 mg/kg/day and greater (157). Despite these increases, only a few patients experienced mild fevers and myalgias at these very high doses.

CYTOKINES At very high doses, i.e., 24 mg/kg/day as a continuous IV infusion, significant increases in IL-6, IL-IRα, and TNFα were observed and often correlated with flu-like syndromes (157). At present, the precise roles of each of the cytokines and complement activation in the clinical signs and symptoms (myalgia and fever) at high doses are not defined.

COAGULATION All phosphorothioate oligodeoxynucleotides studied in normal volunteers and in patients have resulted in transient, self-limited increases in activated partial thromboplastin time (aPTT). The effects are dose and peak plasma concentrations dependent (157). These effects appear to be more prominent after the first dose. In no patients treated have we observed any evidence of bleeding, so the effects on aPTT have not proven to be a problem in the clinic and humans appear to behave similarly to other animals with regard to this side effect.

PLATELET EFFECTS When phosphorothioate oligodeoxynucleotides have been administered by continuous IV infusion in patients with malignant diseases, transient thrombocytoplastin has been observed in a few patients (157). This effect was more frequent during the first course of therapy, was not obviously dose-related, and was not associated with bone marrow effects (158, 162). Most of the time, platelet counts returned to normal while dosing was continued and no bleeding was observed. The mechanism for this effect is not clear but probably involves margination.

THE MEDICINAL CHEMISTRY OF OLIGONUCLEOTIDES

The core of any rational drug discovery program is medicinal chemistry. Although the synthesis of modified nucleic acids has been studied for some time, the intense focus on the medicinal chemistry of oligonucleotides dates to perhaps five years ago. The scope of medicinal chemistry has expanded enormously, but the biological data to support conclusions about synthetic strategies are only beginning to emerge.

The base, sugar, and phosphate moieties of oligonucleotides and oligonucleotide conjugates have been modified. The subjects of medicinal chemical programs include approaches to create stronger and more selective affinity for RNA or duplex structures; to provide the ability to cleave nucleic acid targets; and to enhance nuclease stability, cellular uptake and distribution, and in vivo tissue distribution, metabolism, and clearance.

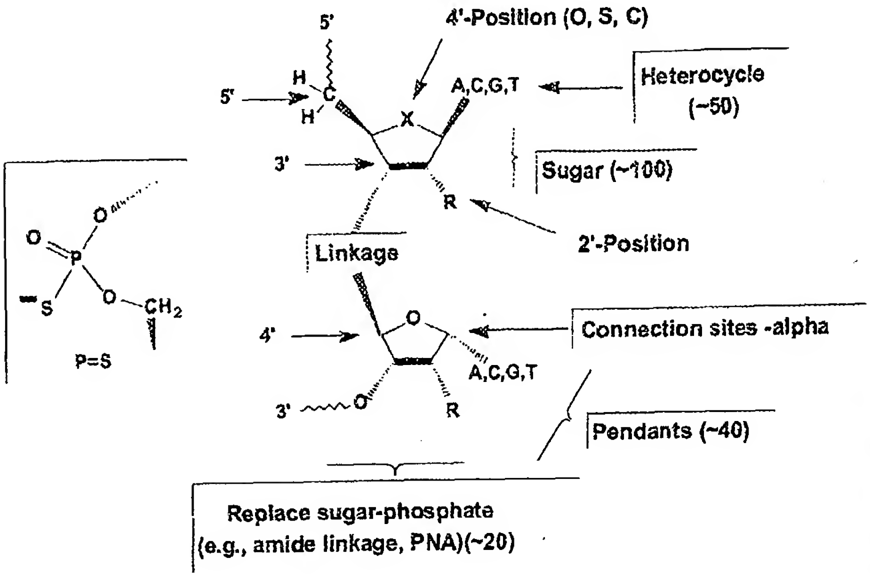

Figure 3 Isis oligonucleotide modifications.

Figure 3 shows a dinucleotide and the sites and type of modifications studied. Perhaps the most fruitful area for modifications has been the 2′ position. Several hundred 2′ modifications have been studied, of which the most interesting is the 2′ methoxyethyl modification (Figure 4).

2′ methoxyethyl chimeric oligonucleotides have significantly higher affinity than phosphorothioates for RNA. They are at least ten times more potent and have an elimination half-life of 25–30 days in all organs and species studied. Further, this modification reduces the proinflammatory effects of oligonucleotides and has supported oral administration in humans (for review see 1). Several antisense drugs based on this chemistry are in clinical trials today.

Phosphate replacement has also been a fruitful area of research. The morpholino replacement has been studied and several drugs in development are based on morpholino chemistry (for review see 163). Additionally, PNAs have been studied extensively (for review see 1).

2′-*O*-(2-METHOXYETHYL) CHIMERAS: SECOND-GENERATION ANTISENSE DRUGS

The 2′-*O*-(2-methoxyethyl) substitution represents a significant advance in antisense therapeutics and the culmination of more than a decade of progress in antisense technology. Oligonucleotides in which every nucleotide contains a

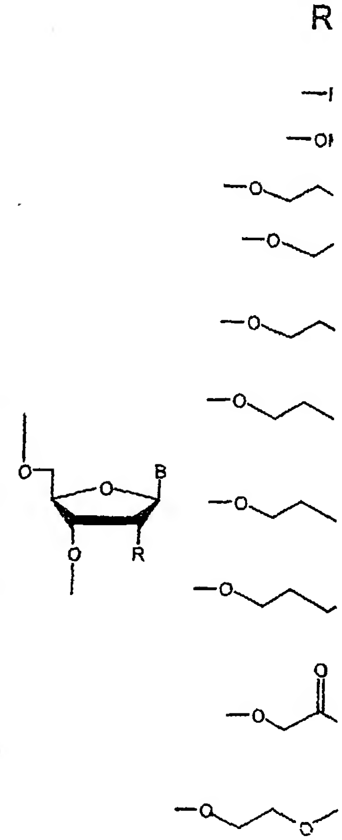

Figure 4 Example of 2′ modific

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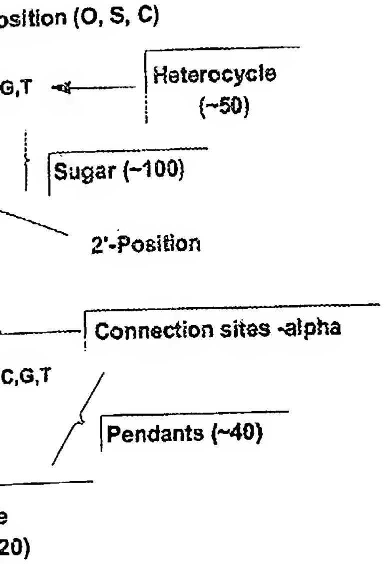

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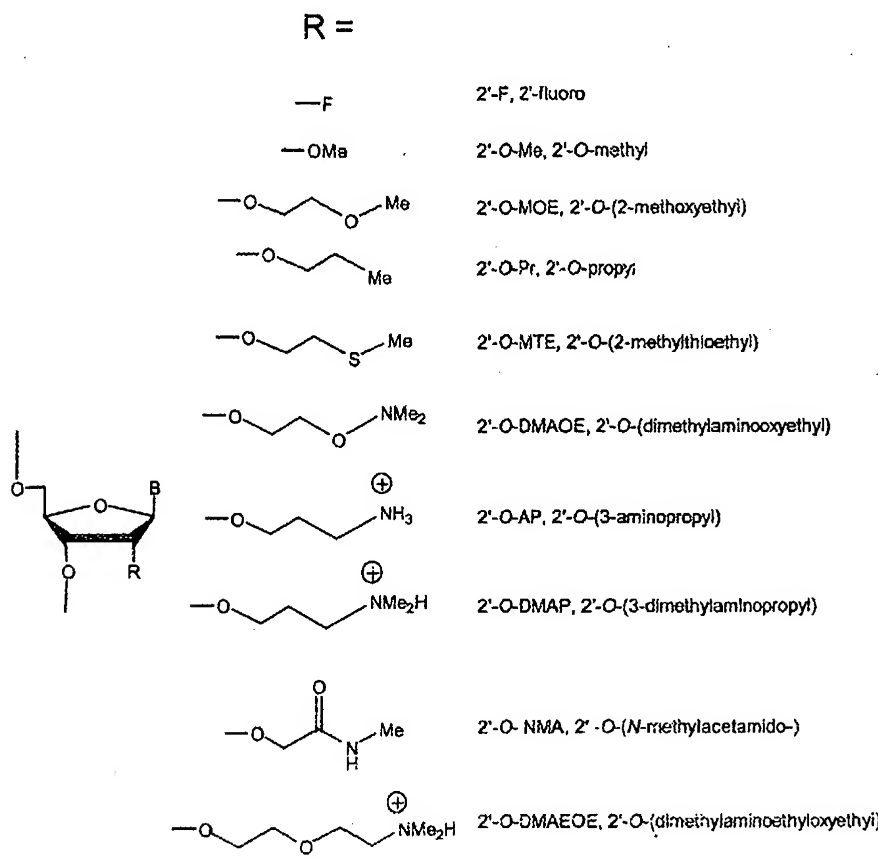

Figure 4 Example of 2′ modification.

2′-*O*-(2-methoxyethyl) modification are of great value when used for mechanisms mediated by occupancy only, e.g., induction of alternative splicing. They have been used most broadly in chimeric structures designed to serve as substrates for RNase H. Numerous chimeric 2′-*O*-(2-methoxyethyl) oligonucleotides have not been studied extensively. Although such oligonucleotides typically display affinity for target RNA that is several orders of magnitude greater than for phosphorothioate oligodeoxynucleotides, they are less attractive substrates for RNase H (for review see 164). Consequently, they typically display only 5–15-fold increases in potency both in vitro and in vivo. For example, a direct comparison of two antisense inhibitors of *c-raf* kinase with the same sequence—one being a phosphorothioate oligodeoxynucleotide, the other a 2′-*O*-(2-methoxyethyl) chimera—showed that in vitro the 2′-*O*-(2-methoxyethyl) chimera was approximately fivefold more potent. Similarly, after IV administration in a rat heart allograph model, the modified

oligonucleotide was at least fivefold more potent (165). Similar data were reported from a direct comparison of antisense inhibitors of survivin in vitro and in vivo in a human tumor xenograft model (166). Pharmacologic studies of antisense oligomers modified with 2′-*O*-(2-methoxyethyl) chemistry have recently been reviewed (167).

Perhaps more importantly, 2′-*O*-(2-methoxyethyl) chimeras are substantially more stable than phosphorothioate oligonucleotides. In mice, rats, and monkeys, the elimination half-life is nearly 30 days in plasma and several tissues (168–171). Furthermore, in the liver, elegant correlations between pharmacokinetic and pharmacodynamic effects have been reported showing a duration of pharmacologic action of nearly 20 days in the mouse (172).

Studies in animals have recently been extended to humans. ISIS 104838, a 2′-*O*-(2-methoxyethyl) chimera that acts as a TNFα antisense inhibitor, reduced TNFα secretion at doses at least tenfold lower than would be expected in humans by first-generation antisense drugs (Dorr, unpublished observations) and to have an elimination half-life in plasma of ~30 days (Geary, unpublished observations). These studies demonstrate the feasibility of monthly dosing.

Despite significant progress in achieving acceptable oral bioavailability of antisense inhibitors, much remains to be accomplished, and important clinical studies now under way will help determine the feasibility of oral delivery (for review see 173). Two key barriers to oral bioavailability have been identified and partially overcome: presystemic metabolism and penetration across the gastrointestinal (GI) mucosa.

Because the gut has a very high level of nucleases contributed by both the host and bacteria resident in the GI tract, metabolism of phosphorothioate oligodeoxynucleotides in the gut occurs much too rapidly to support adequate oral bioavailability (174). Chimeric modifications of the oligonucleotides have been shown to enhance stability and oral bioavailability (175, 176). With the development of 2′-*O*-MOE phosphorothioates, oral bioavailability is potentially feasible (173).

In rodents, dogs, and monkeys, the permeability of the GI tract to antisense inhibitors has been significantly enhanced by formulations containing penetration enhancers such as bile acid salts and fatty acids. After intrajejunal administration of several 2′-*O*-MOE modified oligonucleotides in the presence of penetration enhancer, systemic bioavailability in excess of 20% was observed in all three species (173). Initial studies with solid dose forms containing penetration enhancers resulted in significantly less systemic bioavailability (estimated tissue bioavailability: 10%–15%) (168, 170, 173). Additional clinical trials of various solid dose forms containing ISIS 104838, a 2′-*O*-MOE chimeric antisense inhibitor of TNFα, and various penetration enhancers are in progress, so we should soon have some sense of the near-term potential for oral delivery of antisense inhibitors in humans.

Finally, 2′-*O*-(2-methoxyethyl) chimeras have been shown to be less proinflammatory (46, 177). After repeated subcutaneous dosing in humans, ISIS 104838

produced dramatically l
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ISIS 104838

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LIMITATIONS

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produced dramatically lower local inflammation than first-generation antisense drugs. Thus, more convenient and better locally tolerated subcutaneous administration may be feasible.

ISIS 104838

ISIS 104838 is a second-generation inhibitor of TNF-α. Clinical trials have demonstrated that it reduces in normal volunteers at doses significantly lower than would be required for a first-generation antisense drug. Further, the drug was extremely well tolerated after repeated subcutaneous dosing. Additionally, the study confirmed that second-generation antisense drugs have a long elimination half-life, i.e., 30 days (178).

ISIS 104838 was also reported to be orally bioavailable in humans after administration of solid dose forms. The estimated tissue bioavailability was ~15%. Current studies are optimizing the oral formulations. These formulations would be expected to provide equivalent results for all second-generation 2′ methoxyethyl antisense drugs. A large phase II trial of the subcutaneous form of the drug in patients with rheumatoid arthritis is in progress.

LIMITATIONS

Antisense technology is a versatile and effective tool to evaluate gene function in vitro. In our laboratory, we have made antisense inhibitors to perhaps as many as 4000 genes without failure. Antisense agents are also active in vivo and so can be used to validate drug discovery targets.

The limitations of antisense agents are defined by their pharmacokinetic and toxicologic properties. For systemic therapy, at lower doses, these drugs distribute primarily to liver, kidney, spleen, and fat cells. They do not cross an intact blood-brain barrier and do not seem to enter skeletal muscle. Second-generation antisense drugs display similar distribution properties.

The administration of antisense drugs by alternative routes, such as aerosol, topical, and enema, enhance their potential utility for organs that are not the primary sites of distribution after systemic delivery.

The principal dose-limiting toxicity is proinflammatory effects. In humans, for first-generation antisense drugs, these effects are encountered at doses in excess of 10 mg/kg/day; second-generation antisense drugs are much less prone to cause these side effects.

Antisense drugs have a slow onset of action (24–48 h). This limits their utility in acutely life-threatening diseases such as sepsis and cardiovascular events.

In the future, new approaches that further enhance potency should reduce the impediments to treating some diseases and ease the challenges of oral delivery.

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